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Isolation and characterization of calmodulin from an insulin-secreting tumour

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A major protein constituent of a rat islet cell tumour that exhibited Ca^{2+}-dependent changes in electrophoretic mobility has been purified to homogeneity and compared in its physicochemical and biological properties with bovine brain and rat brain calmodulin (synonymous with phosphodiesterase activator protein, calcium-dependent regulator, troponin C-like protein and modulator protein). The protein, like these calmodulins, contained trimethyl-lysine, exhibited a blocked *N*-terminus and had an identical amino-acid composition and molecular weight on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Peptide 'maps' prepared after digestion of the three proteins with trypsin, papain or *Staphylococcus* V-8 proteinase were virtually superimposable. Ca^{2+} altered the electrophoretic mobilities and enhanced the native protein fluorescence in an equivalent manner with all three proteins. Equilibrium-dialysis experiments demonstrated in each case the binding of 4 g-atoms of calcium/mol of protein; the binding sites were equivalent and showed K_d 0.8 μM. Tumour and brain proteins were equipotent as Ca^{2+}-dependent activators of partially purified rat brain cyclic nucleotide phosphodiesterase, and in this action were inhibited in an identical manner by trifluoperazine. The proteins also exhibited the common property of Ca^{2+}-dependent binding to troponin I, histone H2B and myelin basic protein. The estimated tumour content of calmodulin was 450 mg/kg fresh wt., a value similar to that reported in islets of Langerhans. These results further document the validity of the islet cell tumour as an experimental model of Ca^{2+}-mediated molecular events associated with insulin secretion. They also suggest that brain calmodulin may be substituted for endogenous calmodulin in experimental investigations into the mechanism of insulin secretion.

The secretory response of the pancreatic β-cell to metabolic stimuli depends on extracellular Ca^{2+} (Grodsky & Bennett, 1966; Milner & Hales, 1967) and is inhibited by pharmacological and ionic manipulations perturbing Ca^{2+} transport (Hales & Milner, 1968; Malaisse *et al.*, 1977). Insulin release is accompanied by alterations in the influx and efflux of $^{45}Ca^{2+}$ in islet tissue and by the accumulation of $^{45}Ca^{2+}$ intracellularly particularly in secretory granules and mitochondria (Malaisse-Lagae & Malaisse, 1971; Kohnert *et al.*, 1979). Modulation of the cytosolic free Ca^{2+} concentration, which may be regulated by such changes in Ca^{2+} transport, is thought to be the principal determinant of insulin granule exocytosis (for review see Hedeskov, 1980).

Studies in a variety of tissues have implicated the Ca^{2+}-binding protein, calmodulin, in the mediation of Ca^{2+}-dependent cellular events (for reviews see Cheung, 1980; Klee *et al.*, 1980; Means & Dedman, 1980). The binding of calmodulin to microskeletal elements in the cell (Welsh *et al.*, 1978) and its activation of myosin light-chain kinase (Dabrowska *et al.*, 1978) suggests a role in the control of contractile processes. Its regulatory effects on cyclic nucleotide phosphodiesterase (Kakiuchi & Yamazaki, 1970; Cheung, 1971) and adenylate cyclase (Brostrom *et al.*, 1975) implicate a function in cyclic nucleotide metabolism. Participation in the control of Ca^{2+} transport is suggested by its activation of plasma-membrane $(Ca^{2+} + Mg^{2+})$-dependent ATPase (Jarrett & Penniston, 1978). The ability of calmodulin to activate cyclic AMP-independent protein kinases

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(Waisman *et al.*, 1978) may also have wide-ranging regulatory effects reflected, for example, in changes in plasma-membrane function (Schulman & Greengard, 1978) and in the control of glycogen breakdown (Cohen *et al.*, 1978).

Many of the regulatory effects of calmodulin seem to be of relevance to Ca^{2+}-mediated processes involved in insulin secretion. Exocytosis of secretory granules, for example, is dependent on an intact microtubular/microfilament system (Lacy *et al.*, 1968), is modulated by changes in the cellular concentration of cyclic nucleotides (Charles *et al.*, 1973) and is accompanied by changes in the phosphorylation state of specific proteins (Sugden *et al.*, 1979*a*; Schubart *et al.*, 1980).

The presence of calmodulin in pancreatic islets has been suggested by the partial inhibition of insulin release by a calmodulin antagonist, trifluoperazine {10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine}, and the presence in crude extracts of islets of a heat-stable Ca^{2+}-dependent activator of brain cyclic AMP phosphodiesterase (Sugden *et al.*, 1979*b*). Adenylate cyclase activity in crude islet extracts shows Ca^{2+}-dependent sensitivity to exogenous calmodulin (Valverde *et al.*, 1979). The small amount of tissue available from isolated pancreatic islets has, however, precluded further characterization of the activator in these studies.

Calmodulin is only one of a series of structurally related proteins that mediate in Ca^{2+}-dependent cellular processes; other members, some of which have similar functional properties, include troponin C, myosin light chains, intestinal Ca^{2+}-binding proteins and the parvalbumins (Kretsinger, 1977). Before ascribing a regulatory importance to calmodulin in islet cell function, it is thus necessary to investigate more closely the nature of Ca^{2+}-dependent regulatory proteins in this tissue. The present investigation reports the isolation of such a protein from an islet cell tumour and compares, in parallel studies, its physicochemical and biological properties with those of calmodulin purified from bovine and rat brain. The tumour in question has an insulin content similar to that of isolated pancreatic islets, and ultrastructurally is composed principally of β-cells (Chick *et al.*, 1977). A functional role for calmodulin in the secretory response of this tumour is suggested by the finding that insulin release induced by theophylline and leucine is inhibited by trifluoperazine (Sopwith *et al.*, 1981).

Experimental

Protein purification methods

The rat islet cell tumour, which had initially been induced by X-irradiation (Chick *et al.*, 1977), was propagated by the subcutaneous implantation of 50–100mg of minced tissue into inbred rats (200g body wt.) of the New England Deaconess Hospital (NEDH) strain. Tumours (0.2–2g wet wt.) and whole brains were removed from each animal 4–6 weeks after implantation when animals exhibited symptoms of hypoglycaemia. The tissue was stored at −20°C and processed in quantities of 50–100g. Bovine brains were obtained from the local abattoir within 30min of slaughter and processed immediately. All protein isolation procedures were performed at 4°C unless specified.

Ca^{2+}-sensitive proteins

Tissues were homogenized for 2min in a Waring blender in 4vol. of 100mM-NH_4HCO_3 (pH7.9) containing 2mM-EGTA (BDH Chemicals, Poole, Dorset, U.K.) and 0.5mM-phenylmethanesulphonyl fluoride [Sigma (London) Chemical Co.]. The homogenate was then sonicated (medium setting of an MSE ultrasonic disintegrator Mk2; MSE, Crawley, Sussex, U.K.), filtered through cheesecloth and centrifuged for 30min at 88000*g*. The supernatant was mixed for 20min at 20°C with DEAE-cellulose (DE-52; Whatman, Springfield Mill, Kent, U.K.) that had been equilibrated in the homogenization buffer (1g of wet cake/g of tissue).

The DEAE-cellulose was recovered by vacuum filtration, washed with 2 litres of homogenizing buffer and packed into a 2.5cm diameter column (5cm for the beef brain preparation), which was then eluted at 2.5ml/min with 10 bed vol. of a linear 0.1–0.8M-NH_4HCO_3 gradient containing 2mM-EGTA. The protein was identified in eluted fractions by gel electrophoresis at pH8.6. Fractions containing the protein (conductivity 16–19mS) were combined, dialysed overnight against water and freeze-dried. The freeze-dried material was dissolved in water (5–20ml), centrifuged for 30min at 40000*g* to remove insoluble material and 7.5ml portions were chromatographed on a column (2.5cm × 1m) of Sephadex G-75 Superfine (Pharmacia, Uppsala, Sweden) with an equilibration and elution buffer of 100mM-NH_4HCO_3 containing 0.1mM-EGTA (flow rate 0.2ml/min). Fractions containing the protein (V_e 245ml) were applied directly on a column (1.6cm × 30cm) of DEAE-Sephadex A-25 (Pharmacia), which was then eluted with a 1 litre linear gradient of 0.2–0.8M-NH_4HCO_3 (flow rate 4.2ml/min). The protein (elution conductivity 21–23mS) was freeze-dried and then chromatographed on a column (2.5cm × 15cm) of hydroxyapatite (Bio-Gel HTP; Bio-Rad, Richmond, CA, U.S.A.) with a 1 litre linear gradient of 5–100mM-sodium phosphate at pH6.8 (flow rate 0.5ml/min). Fractions containing the protein were combined, dialysed against 5mM-NH_4HCO_3 (pH7.9) containing 0.1mM-EGTA then against 5mM-NH_4HCO_3 (pH7.9) and finally freeze-dried and stored at

−70°C. Protein concentration was determined by the Folin reaction (Lowry *et al.*, 1951) with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard.

Calmodulin-sensitive phosphodiesterase

Cyclic nucleotide phosphodiesterase (3′:5′-cyclic nucleotide 5′-nucleotidohydrolase; EC 3.1.4.17) was isolated in conjunction with the rat brain Ca^{2+}-sensitive protein. Activity detected in the eluate of the DEAE-cellulose column (conductivity 7–10 mS) was concentrated by freeze-drying and then subjected to $(NH_4)_2SO_4$ precipitation, Sephadex G-100 gel filtration and affinity chromatography as described by Klee & Krinks (1978). The affinity support was prepared from the above bovine brain Ca^{2+}-sensitive protein reacted with CNBr-activated Sepharose (Pharmacia) (1 mg of protein/ml bed volume).

Enzyme assays were routinely performed at 30°C in 1 ml of buffer, pH 7.5, containing 30 mM-Tris/HCl, 30 mM-imidazole, 18 mM-magnesium acetate, 0.15 mM-$CaCl_2$, 0.1 mM-dithiothreitol, 0.15 mM-cyclic AMP, 0.4 unit of *Crotalus atrox* venom 5′-nucleotidase/ml (EC 3.1.3.5) (Sigma), 0.05 unit of calf thymus adenosine deaminase/ml (EC 3.5.4.4) (Sigma) and 1.5–3 μg of extracted phosphodiesterase protein/ml. The Ca^{2+}-sensitive proteins, when included, were added from a 200-fold concentrated solution in the reaction buffer. Trifluoperazine dihydrochloride (Smith, Kline and French, Welwyn Garden City, Herts., U.K.) was added from freshly prepared unbuffered aqueous solutions stored in light-proof containers.

The reaction was monitored for from 2 to 10 min at 265 nm in 0.5 cm-light-path cuvettes with a Gilford model 250 spectrophotometer (Gilford Instrument Co., Oberlin, OH, U.S.A.). A value of 7.02×10^6 litre·mol^{-1}·cm^{-1} for the difference between the extinction coefficients of cyclic AMP and the final reaction product inosine was used in calculating the results. Absorbance changes were linear with respect to time and enzyme concentrations within the ranges used.

Other proteins

Myelin basic protein was purified from post-mortem human brain by the method of Deibler *et al.* (1972).

Rabbit skeletal-muscle troponin C and troponin I were gifts from Dr. J. M. Wilkinson, Department of Biochemistry, University of Birmingham, Birmingham, U.K. Calf thymus histone H2B (type VIIs) was obtained from Sigma.

Electrophoresis techniques

Column elution profiles and investigations of the interactions of the Ca^{2+}-sensitive protein with other purified proteins were performed by electrophoresis on 7.5% polyacrylamide slab gels (15 cm × 15 cm) containing 6 M-urea in a continuous buffer system of 28 mM-Tris base and 190 mM-glycine, pH 8.6. Samples (50 μl) applied to the gels contained 75 mM-Tris/HCl, pH 8.9, 5 M-urea, 0.05% (w/v) Bromophenol Blue, 15 mM-mercaptoethanol and either 1 mM-$CaCl_2$ or 1 mM-EGTA. Tissue homogenates were usually made directly in sample buffer (tissue/volume ratio 1:5), then centrifuged at 4°C for 20 min at 88000 *g* and filtered through glass wool to remove insoluble material. In some cases tissue was homogenized in buffer without urea, heated for 2 min at 95°C, and the supernatant obtained after centrifugation (5 min at 9000 *g*) was subjected to electrophoresis after addition of urea to a concentration of 5 M.

Molecular-weight determinations and analysis of proteolytic digests were performed on 15% polyacrylamide slab gels (15 cm × 15 cm) containing 0.1% (w/v) sodium dodecyl sulphate by the method of Laemmli (1970). A mixture of bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c* was used as a molecular-weight calibration standard.

Amino-acid composition analyses

Protein hydrolysates were usually prepared by heating samples (50–100 μg/ml) for 20 h at 110°C under vacuum in 6 M-HCl containing 1 mM-phenol (Sanger & Thompson, 1963). Tryptophan determinations were performed, however, after hydrolysis for 20 h at 110°C under vacuum in 2 M-methanesulphonic acid containing 0.2% (w/v) 3-(2-aminoethyl)indole (Pierce Chemical Co., Rockford, Il. U.S.A.) (Simpson *et al.*, 1976). The same conditions were used for cystine and cysteine determinations after conversion of these amino acids into *S*-sulphopropyl derivatives with tri-n-butyl phosphine (Koch–Light, Colnbrook, Bucks., U.K.) as a reductant and 3-hydroxypropane-1-sulphonic acid (Feinbiochemica, Heidelberg, Germany) as an alkylating agent (Rüegg & Rudinger, 1977).

Qualitative analysis of *N*-terminal amino acids was achieved by dansylation of the protein followed by hydrolysis in HCl (see above) and chromatographic separation of the products on Cheng Chin polyamide plates (5 cm × 5 cm; BDH Chemicals) (Hartley, 1970). Trimethyl-lysine was estimated in an identical manner, except that hydrolysis was performed before dansylation. Trimethyl-lysine standard was obtained as the bis-(*p*-hydroxyazobenzenesulphonate) salt from Calbiochem. San Diego, CA, U. S.A.

Proteolytic digest 'mapping'

Tryptic peptides were prepared by incubation of 50 μg of the proteins for two successive 3 h periods

at 37°C in 200μl of 1% NH_4HCO_3, pH 7.9, to which each time was added 0.25μg of 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated trypsin (Worthington, Freehold, NJ, U.S.A.) (Jackson *et al.*, 1975). Peptides were separated on Polygram CEL300 0.1mm cellulose plates by electrophoresis at pH 2 or pH 6.5 in the first dimension followed by ascending chromatography in the second, and then detected with a fluorescamine {4-phenylspiro[furan-2(3*H*),1-phthalan]-3',3'-dione} solution (0.01%, w/v) in 2% (v/v) pyridine in dry acetone.

Digests of protein (10μg) samples with 2μg quantities of papain (EC 3.4.22.2; Boehringer Mannheim) or *Staphylococcus aureas* V-8 proteinase (Miles Laboratories, Slough, Bucks., U.K.) were performed for 30min at 37°C in 100μl of a buffer, pH 6.8, containing 125mM-Tris, 0.5% (w/v) sodium dodecyl sulphate and 10% glycerol. These were then electrophoresed on 15% polyacrylamide/sodium dodecyl sulphate gels and detected with Coomassie Blue staining (Cleveland *et al.*, 1977).

Spectroscopic measurements

U.v.-absorption spectra were recorded at 20°C with a Zeiss DM21 spectrophotometer (Zeiss U.K. Ltd., Borehamwood, Herts., U.K.) from solutions of the Ca^{2+}-sensitive proteins (1mg/ml) in 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate] buffer, pH 7.4. Fluorimetric data (uncorrected) were obtained at 20°C with an Aminco–Bowman spectrophotofluorimeter (American Instrument Co., Silver Springs, MD, U.S.A.) from protein solutions (0.1mg/ml) in 50mM-Hepes buffer, pH 7.4, to which was added 0.1mM-EGTA and various concentrations of $CaCl_2$ (0.01–0.2mM). The resultant concentration of ionized Ca^{2+} in these solutions was calculated by the method of Portzehl *et al.* (1964) taking into account both the added $CaCl_2$ and the Ca^{2+} contamination. The latter (<10μM) was determined by atomic-absorption spectroscopy (Unicam SP-90; Pye, Cambridge, U.K.) after precipitation of the protein in the sample with 15% trichloroacetic acid containing 1% $LaCl_3$. Solutions used in these and in $^{45}Ca^{2+}$-binding studies were prepared in double-glass-distilled water treated with bivalent-cation-chelating resin (Chelex 100; Bio-Rad). Standard $CaCl_2$ solutions were prepared from $CaCO_3$ and HCl; EGTA solutions were titrated against these with murexide as a metallo-indicator (Chaberek & Martell, 1959). Glassware was rinsed in 0.1M-HCl before use.

$^{45}Ca^{2+}$ binding determinations

Equilibrium-dialysis chambers were constructed from polystyrene tubes (6.2cm × 0.8cm LP3 tubes; W. Sarstedt, Leicester, U.K.), their polythene caps and 2cm squares of moistened dialysis tubing (Visking; Medicell International, London, U.K.). Protein samples (10μg in 15μl of water) were pipetted in the chamber formed by the cap flange and 2ml of the equilibrium buffer into the tube; dialysis membrane was then placed over the tube mouth and the cap was inserted. The tube was centrifuged at 20°C for 30s at 100*g* to transfer the protein solution on to the dialysis membrane interface. The tube was then inverted and allowed to stand for 24h at 4°C with intermittent mixing. To recover the dialysed sample, the tube was centrifuged for 30s at 100*g* in an inverted position and the cap was removed. Water (200μl) was then added and samples were removed for determination of radioactivity by liquid-scintillation counting with a Triton X-100-based fluor 'cocktail' (scintillation mix T; Hopkin and Williams, Chadwell Heath, Essex, U.K.). Protein concentration was determined fluorimetrically (Undenfriend *et al.*, 1972). The dialysis buffer contained 50mM-Hepes, pH 6.8 or 7.4, 5μCi of ^3H_2O/ml, 1μCi of $^{45}CaCl_2$/ml (40mCi/mg of calcium; The Radiochemical Centre, Amersham, Bucks., U.K.), 0.1mM-EGTA and various concentrations of $CaCl_2$. The ^3H found in the dialysed sample represented the volume of protein solution recovered (about 85% of the initial) and the ^{45}Ca found in excess of that predicted from the $^{45}Ca/^3H$ ratio in the equilibrium buffer represented the ^{45}Ca bound to protein.

Derivation of thermodynamic and kinetic constants

The dissociation constants for Ca^{2+} binding were obtained from Scatchard (1949) plots. K_m and $V_{max.}$ values for phosphodiesterase were derived from analysis of the data as recommended by Hofstee (1959) and inhibitor constants for trifluoperazine from extrapolation of plots of the reciprocal of initial reaction velocity against inhibitor concentration. Fluorescence enhancement by Ca^{2+} and phosphodiesterase activation by calmodulin were plotted against the logarithm of the protein concentration to obtain values of the effector concentration producing a half-maximal response. In all the above instances at least six intermediate data values were used in the analyses.

Results

Ca^{2+}-sensitive proteins in crude tumour extracts

Electrophoretic analyses of crude tumour extracts demonstrated a major protein component that behaved in a Ca^{2+}-dependent fashion (Fig. 1). A fast-running protein band was observed on 7.5% polyacrylamide gels when the sample was prepared with 1mM-EGTA, but not if 1mM-$CaCl_2$ was included in the homogenization buffer. Supernatant fluid obtained after heat treatment (95°C for 2min) of the tissue homogenate contained a protein with

identical mobility to that seen above with EGTA. However, a band of the same intensity and mobility was still evident when Ca^{2+} was present. This was also the case when tumour was homogenized in 50% methanol and the soluble components were electrophoresed (results not shown). Such behaviour was consistent with the protein being similar to calmodulin or troponin C (Grand *et al.*, 1979); it could be distinguished from the latter, however, by its slightly higher mobility.

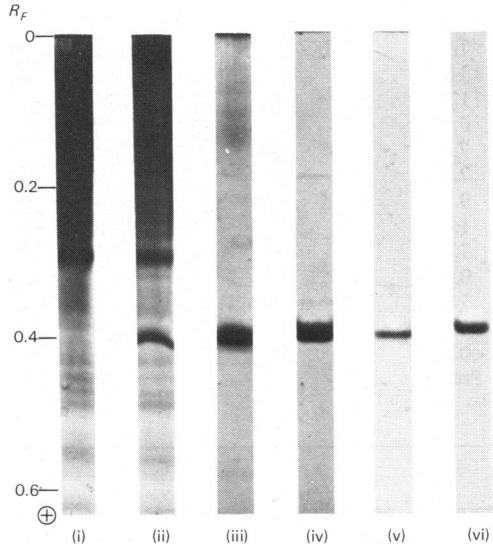

Fig. 1. *Purification of the rat tumour protein*
Samples (50 μl) were subjected to electrophoresis on 7.5% polyacrylamide-gels containing 6 M-urea, 28 mM-Tris and 190 mM-glycine. Samples were as follows: (i) crude tumour extract + 1 mM-$CaCl_2$; (ii) crude tumour extract + 1 mM-EGTA; (iii) DEAE-cellulose chromatography fraction; (iv) Sephadex G-75 fraction; (v) DEAE-Sephadex fraction; (vi) hydroxyapatite fraction. The electrophoretic mobility relative to that of Bromophenol Blue is indicated.

Purification of Ca^{2+}-sensitive proteins from tumour and brain

The chromatographic procedures used to isolate the Ca^{2+}-sensitive protein from tumour and brain were similar to those used in the purification of calmodulin from other tissues (Watterson *et al.*, 1976; Jarrett & Penniston, 1978; Grand *et al.*, 1979). In the present protocol, however, treatment with heat, low pH, organic solvent or urea was avoided, so as to minimize possible modification of the native structure. The Ca^{2+}-sensitive protein from tumour migrated as a single band on urea gels, pH 8.6 (Fig. 1) or sodium dodecyl sulphate gels. The apparent molecular weight on the latter system was 16900, either in the presence or absence of 1% mercaptoethanol. Attempts to identify an *N*-terminal amino acid were unsuccessful and thus suggestive of a blocked *N*-terminus. This further indicated the purity and monomeric nature of the protein. The yield and degree of purification achieved at each step in the isolation procedure shown in Table 1.

The chromatographic behaviour of Ca^{2+}-sensitive proteins of rat and beef brain during isolation were identical with that of the tumour protein. The isolated proteins from these sources, like the tumour protein, exhibited blocked *N*-termini and showed the same mobilities on urea and sodium dodecyl sulphate gel systems. Rat brain yielded 198 mg of Ca^{2+}-sensitive protein/kg wet wt., beef brain 96 mg/kg and rat tumour 66 mg/kg.

Structural analyses

The amino-acid compositions of the three isolated proteins (Table 2) were indistinguishable and in close agreement with the reported composition of beef brain calmodulin (Watterson *et al.*, 1980). The proteins could be distinguished from troponin C (Collins *et al.*, 1973) by their higher content of threonine and the absence of cysteine residues.

The unusual amino acid, trimethyl-lysine, which is characteristic of several mammalian calmodulins (Klee *et al.*, 1980), was demonstrated in all three

Table 1. *Purification and yield of rat tumour protein*
Frozen tumour (50 g) was subjected to the purification procedure outlined in the Experimental section. A final yield of 3.3 mg was attained.

		Ca^{2+}-sensitive protein	
Fraction	Total protein (mg)	Yield (%)	Purification factor
Initial homogenate	7911.0	100	1
Supernatant (30 min at 88000 *g*)	5640.0	100	1.5
DEAE-cellulose eluate	118.5	75	125
Sephadex G-75 eluate	8.1	67	649
Sephadex A-25 eluate	5.1	48	740
Hydroxyapatite eluate	3.3	31	741

Table 2. *Amino-acid composition of the isolated proteins*

Results shown were obtained from three or four analyses of the protein hydrolysates prepared with either HCl or methanesulphonate. Calculations assumed 147 amino-acid residues/molecule, a value derived from the indicated composition of beef brain calmodulin excluding trimethyl-lysine (Watterson *et al.*, 1980). The composition of rabbit skeletal-muscle troponin C is based on the sequence published by Collins *et al.*, (1973).

	Amino-acid content of Ca^{2+}-sensitive proteins (residues/molecule)			Predicted amino acid content (residues/molecule)	
Amino acid	Bovine brain	Rat brain	Islet cell tumour	Calmodulin	Troponin C
Cys + ½CyS	<0.5	<0.5	<0.5	0	1
Asx	22.3	22.8	22.3	23	22
Thr	11.5	11.4	11.2	12	6
Ser	4.9	4.8	5.4	4	7
Glx	27.4	26.2	27.9	27	31
Pro	2.0	1.9	1.9	2	1
Gly	13.2	12.5	13.5	11	13
Ala	11.7	11.6	11.3	11	13
Val	6.9	6.9	6.9	7	7
Met	5.6	7.7	7.3	9	10
Ile	7.6	7.6	7.7	8	9
Leu	9.4	9.6	9.4	9	9
Tyr	2.0	1.9	1.6	2	2
Phe	7.9	8.1	7.5	8	10
His	1.0	1.0	0.9	1	1
Lys	7.2	7.6	7.0	7	9
Arg	6.0	6.0	5.7	6	7
Trp	<0.5	<0.5	<0.5	0	0
Trimethyl-lysine	Present	Present	Present	1	0

proteins by two-dimensional t.l.c. after hydrolysis and dansylation. Troponin C, which has a similar amino-acid composition but does not contain trimethyl-lysine, gave a negative test in this procedure.

Tryptic digests of the three proteins subjected to electrophoresis at pH 6.5 followed by partition chromatography in the second dimension showed eleven peptide products, all with similar relative positions for each protein digest (Fig. 2). When electrophoresis was performed at pH 2.0 the peptide 'maps' were again virtually superimposable. However, rat brain and rat tumour proteins gave 20 peptides, whereas 21 peptides were observed in beef brain protein digests. This additional peptide may have been present in the other preparations as evidenced, at least in the rat brain digest, by an asymmetry of the peptide spot adjacent to the position of the unique peptide in the beef brain protein digest. Identical tryptic peptide 'maps' have been reported for calmodulins purified from the brains of beef, rats, pigs, rabbits and chickens (Vanaman *et al.*, 1976).

Electrophoretic analyses of the peptide products formed by digestion of the Ca^{2+}-sensitive proteins with papain or *Staphylococcus* V8 proteinase yielded patterns that were quite different for each proteinase; nevertheless, the three Ca^{2+}-sensitive proteins gave identical patterns (results not shown).

Spectroscopic analyses

The u.v.-absorption spectra of the three proteins isolated were characterized by absorption maxima at 253, 259, 265, 269 and 276 nm. This property, which may be attributed to the absence of tryptophan and a high ratio of phenylalanine to tyrosine (Table 2), is a feature of calmodulin isolated from various sources (Klee *et al.*, 1980).

All three proteins showed characteristic changes of their native fluorescence in the presence of Ca^{2+} (Table 3, Fig. 3). Increase of the free ionized Ca^{2+} concentration within the range of 10 nM to 10 μM, as controlled by a Ca^{2+} EGTA buffer system, resulted in a progressive enhancement in the native fluorescence of the proteins. The wavelengths at which excitation and emission maxima were recorded were not affected by the Ca^{2+} concentration. The magnitude of the Ca^{2+}-induced change was similar for each of the three proteins and occurred at comparable free Ca^{2+} concentrations (Table 3).

Ca^{2+} binding

Equilibrium-dialysis data obtained at either pH 6.8

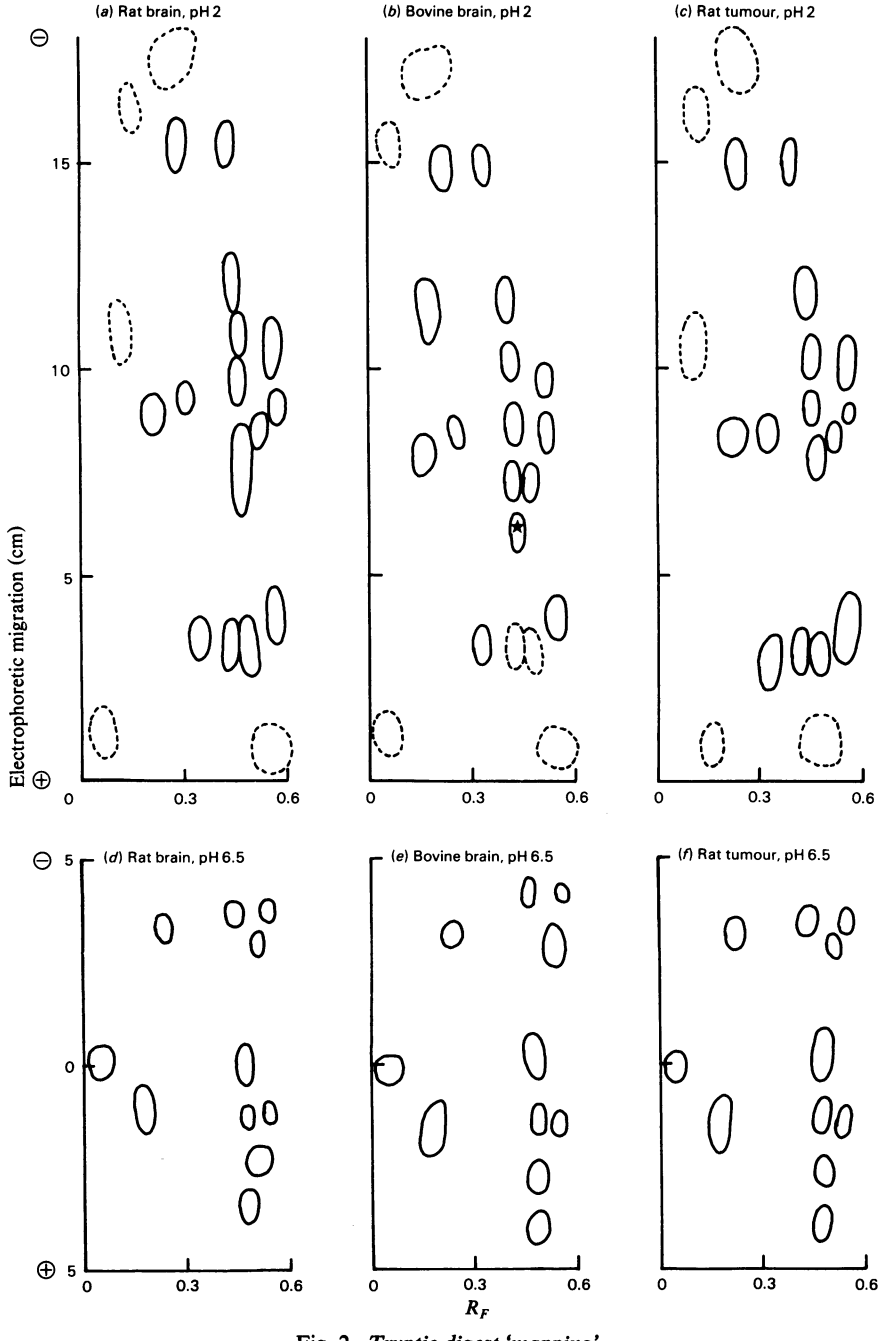

Fig. 2. *Tryptic digest 'mapping'*

Tryptic digests were subjected to electrophoresis in the first dimension either at pH 2 or 6.5 and then chromatographed. The point of sample application is marked by the null co-ordinate. Less prominent tryptic peptides are shown by a broken outline. A unique peptide observed in beef brain protein digests is indicated by the asterisk.

or 7.4 showed that the Ca^{2+}-sensitive proteins isolated possessed four equivalent Ca^{2+}-binding sites each with a dissociation constant of about 1 μM (Fig. 3, Table 3). The three proteins were indistinguishable on these criteria. Similar values for the dissociation constant for calmodulin–Ca^{2+} com-

Table 3. *Ca^{2+}-induced changes in native fluorescence and Ca^{2+} binding*

Fluorescence measurements (λ excitation 276nm, λ emission 324nm) are shown for proteins in the presence of 0.1mM-EGTA (F_0) or 0.1mM-EGTA plus 0.1mM-$CaCl_2$ (F_1). The Ca^{2+} concentrations at which half-maximal changes in fluorescence were observed ($[Ca]F_{50}$) were derived from titration data similar to the results shown in Fig. 3(*a*). Results of equilibrium-dialysis determinations were derived from Scatchard analyses of data similar to the results shown in Fig. 3(*b*). The maximal binding and apparent dissociation constant for calcium (K_d) are shown as means of estimates at pH6.8 and 7.4. A molecular weight of 16700 is assumed for each protein.

	Fluorescence (arbitrary units)			Calcium binding	
	F_0	F_1	$[Ca]F_{50}$ (M)	(g-atom/mol of protein)	K_d (M)
Bovine brain	13.2	19.8	1.7×10^{-7}	3.7	1.0×10^{-6}
Rat brain	13.2	18.4	1.3×10^{-7}	3.9	0.9×10^{-6}
Rat tumour	17.7	21.4	1.1×10^{-7}	3.9	0.8×10^{-6}

plexes have been reported by Dedman *et al.* (1977) with a similar Ca^{2+}/EGTA buffer system. In general, however, published values for the dissociation constant are at variance (Klee *et al.*, 1980).

Comparison of the fluorescence enhancement and Ca^{2+}-binding data (Figs. 3*a*–3*c*) showed that the major change in fluorescence of the Ca^{2+}-sensitive proteins occurred on binding of the first two calcium atoms. Similar observations have been made with regard to the Ca^{2+}-dependency of the u.v.-absorption and circular-dichroism properties of beef brain calmodulin and suggest that the major conformational transition in the molecule thought responsible for these spectroscopic properties occurs at less than full occupancy of its Ca^{2+}-binding sites (Klee, 1977).

Phosphodiesterase activation

The rat brain phosphodiesterase prepared in the present investigation when analysed by sodium dodecyl sulphate gel electrophoresis showed a major protein component of mol.wt. 60400 and a minor one of mol.wt. 115000.

The Ca^{2+}-sensitive proteins purified from tumour and brain provoked an immediate (less than 10s) increase in phosphodiesterase activity (Fig. 4, Table 4) equivalent to 3–15 times that of the basal catalytic rate. This activation was attributable to a large increase in $V_{max.}$ accompanied by a small decrease in the K_m for cyclic AMP from 75 to 48μM. The concentration of Ca^{2+}-sensitive protein that caused half-maximal activation was similar for each of the three proteins studied.

Heating of a 0.1mg/ml solution of the tumour Ca^{2+}-sensitive protein did not affect its ability to activate the phosphodiesterase. Such treatment, however, denatured endogenous phosphodiesterase in tumour homogenates. It was thus possible to assay for the activator from the capacity of appropriately diluted heat-treated homogenates to activate the purified enzyme. The initial tumour content of phosphodiesterase activator determined as equivalent to the Ca^{2+}-sensitive protein was 23.1 ± 0.7 μmol/kg wet wt. ($n = 4$) or approx. 0.4% of the total tumour protein content.

Activation of phosphodiesterase by the tumour or brain proteins was markedly decreased when the free Ca^{2+} concentration was decreased to 0.1μM or less by the addition of EGTA (Table 4). The effect was fully reversible on further addition of $CaCl_2$ to attain a free Ca^{2+} concentration of 10μM. The basal enzyme activity, in contrast with this, was only slightly affected by changes in Ca^{2+} concentration.

The calmodulin antagonist trifluoperazine had little effect on basal phosphodiesterase activity, but inhibited the activation of the enzyme by the three Ca^{2+}-sensitive proteins. In each case the inhibitor constants determined in the presence of maximally effective concentrations of the activator were in the range of 5–10μM. Binding data suggest that calmodulin possesses two high-affinity sites for trifluoperazine with dissociation constants of about 1μM (Weiss & Levin, 1977), a finding compatible with the present data.

Formation of complexes with other purified proteins

In the presence of Ca^{2+}, the tumour Ca^{2+}-sensitive protein formed a complex with myelin basic protein, histone H2B and troponin I. This was evidenced by the disappearance of the band corresponding to the Ca^{2+}-sensitive protein on electrophoresis and the formation of a more slowly moving protein band (results not shown).

When samples were prepared with EGTA present, complex-formation was either abolished, or at least markedly curtailed. A slight increase in the mobility of the tumour protein was observed when electrophoresed by itself in the presence of Ca^{2+}. Bovine brain and rat brain Ca^{2+}-sensitive proteins behaved in an identical manner with that of the tumour protein with respect to all these criteria. The results

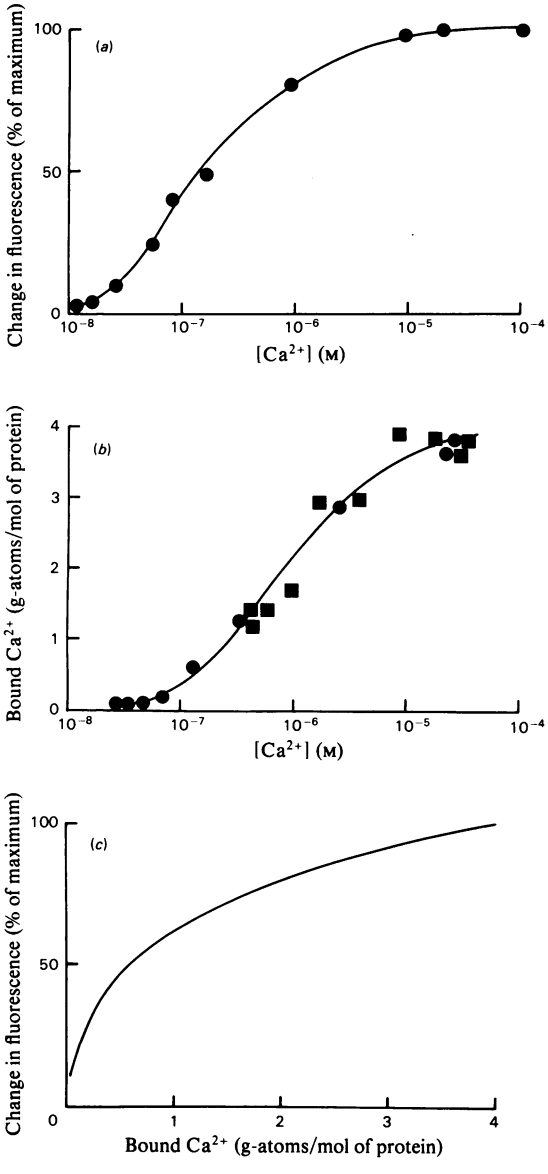

Fig. 3. *Ca^{2+}-dependent changes in the native fluorescence and Ca^{2+}-binding of the purified rat tumour protein*

(*a*) Native fluorescence (λ excitation 276nm, emission 324nm) was determined in response to incremental changes in the $CaCl_2$ content in a buffer containing initially 0.1mM-EGTA. Results are expressed as percentages of the total fluorescence change observed (see Table 3). Each plotted value is the mean for three separate titrations. (*b*) Ca^{2+} binding determined by equilibrium dialysis at pH6.8 (■) and pH7.4 (●) was calculated assuming a molecular weight of 16700. Each plotted value is the result of an individual incubation. (*c*) The relationship of fluorescence changes to Ca^{2+} binding is derived from interpolation of the data shown in (*a*) and (*b*).

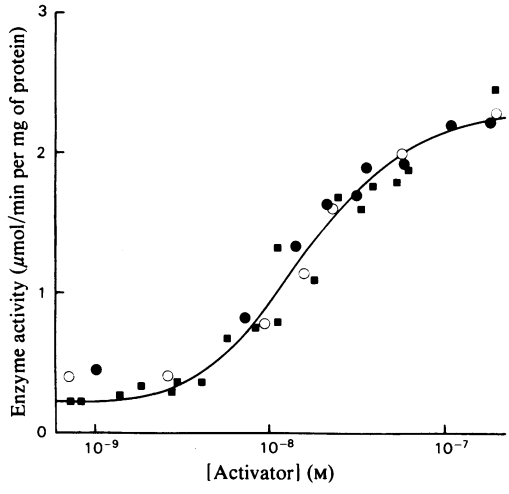

Fig. 4. *Concentration dependency of phosphodiesterase activation by Ca^{2+}-sensitive proteins*

Enzyme activities were determined in the presence of 0.15mM-$CaCl_2$ in experiments in which reaction rates were measured in response to incremental changes in the protein activator concentration. The source of the proteins was bovine brain (■), rat brain (●) and rat tumour (○).

obtained concord with those of Grand & Perry (1980), who originally described such interactions using proteins purified from bovine brain.

Discussion

Calmodulin has been identified in a wide range of eukaryote organisms, including the vertebra, plants, fungi and protozoa. Partial amino-acid sequences of the protein from bovine uterus (Grand & Perry, 1978) and rat testis (Dedman *et al.*, 1978) show remarkable homology with the structure of bovine brain calmodulin (Watterson *et al.*, 1980), indicative of a high degree of conservation during evolution.

The protein presently isolated from a pancreatic islet cell tumour possessed physicochemical and biological properties that identified it as calmodulin. It could be distinguished from the closely related protein, troponin C, by several criteria including its amino-acid composition, the presence of four high-affinity Ca^{2+}-binding sites, its capacity to activate cyclic AMP phosphodiesterase at a low concentration and the inhibition of its action by trifluoperazine. When compared in paired experiments with a calmodulin purified from rat brain it was identical. Except for a minor difference in its tryptic peptide 'map' it could not be distinguished from bovine brain calmodulin on any qualitative or quantitative basis. These findings lend support

Table 4. *Activation of rat brain phosphodiesterase*

Enzyme activities were initially determined in buffer containing 0.1 mM-EGTA, then after the addition of 0.25 mM-$CaCl_2$ and finally after 40 μM-trifluoperazine was added. Activator protein was used at 5 μg/ml, the enzyme at 1.5 μg/ml. The activator concentration required for half-maximal activation (K_a) was determined in the presence of 0.15 mM-$CaCl_2$ from data such as those shown in Fig. 4. The K_i for trifluoperazine was determined by a similar protocol using an activator concentration of 5 μg/ml. Each value is the mean of duplicate or triplicate determinations.

	Enzyme activity (μmol/min per mg of protein)				
Addition	EGTA	Ca^{2+}	Trifluoperazine	K_a (M)	K_i (M)
None	0.61	0.94	1.04		
Bovine brain activator	0.42	4.27	1.14	1.7×10^{-8}	10.0×10^{-6}
Rat brain activator	0.58	3.83	1.08	2.0×10^{-8}	10.0×10^{-6}
Rat tumour activator	0.68	3.92	1.12	1.7×10^{-8}	6.5×10^{-6}

to the emerging concept of calmodulin being highly conserved not only physicochemically but also functionally, and of its being widely distributed with little or no tissue variability.

The tumour content of calmodulin was comparable with that reported in isolated islets of Langerhans (Sugden *et al.*, 1979*b*; Valverde *et al.*, 1979). This observation and the results of studies on the secretory properties of the tumour (Sopwith *et al.*, 1981) suggest this tissue to be a useful model to investigate the mechanism of insulin secretion. The identification of a major protein constituent with properties indistinguishable from those of brain calmodulin constitutes an initial step towards the description of insulin secretion at the molecular level. It also validates the application of brain calmodulin in experimental studies with the tumour and in experiments involving isolated islets.

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