THE STIMULATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY A M_r 11 500 CALCIUM BINDING PROTEIN FROM HEPATOMA

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1. Introduction

A low molecular weight, calcium binding protein has been located in several hepatomas [1], and purified to homogeneity [2]. The tumour calcium-binding protein (tumour CaBP) binds 2 calcium atoms/molecule, has an isoelectric point of 3.9, and is M_r 11 500 [2]. It cannot be located chemically [1] or immunoelectrophoretically [3] in any normal tissue. The N-terminus is blocked, and the amino acid composition is similar to parvalbumin, a M_r 11 500 calcium-binding protein from muscle [2]. However comparison with rat parvalbumin shows the two to be different (unpublished).

Calmodulin (CaM) is a larger (M_r 16 700) ubiquitous calcium-binding protein which activates many different enzymes, including cyclic nucleotide phosphodiesterase (reviews [4–7]). Amino acid sequence data show this protein to be highly conserved throughout plant and animal evolution, and to belong to the troponin C superfamily [8], which also includes parvalbumin.

There have been reports that troponin C and parvalbumin can substitute for CaM in activation of some CaM-dependent enzymes [9,10], although contamination of these muscle proteins with CaM has been suggested [4,11]. This communication demonstrates that the tumour CaBP was neither contaminated with CaM nor a fragment of CaM, yet stimulated a CaM-dependent enzyme in a calcium-dependent manner.

2. Materials and methods

Tumour CaBP from rat hepatoma [2], CaM from rat testes [12], and parvalbumin from rat skeletal muscle (unpublished) were purified to homogeneity (fig.1). Cyclic [^{3}H]guanosine 3′,5′ monophosphate (17 Ci/mmol) was from New England Nuclear (Dorval, P. Q.), and was purified by AG-IX-8 (BioRad Labs., Mississauga, Ont.) chromatography before use. [^{125}I]-Bolton-Hunter reagent (1375 Ci/mmol) was from Amersham (Oakville, Ont.). DEAE-Sephacel,

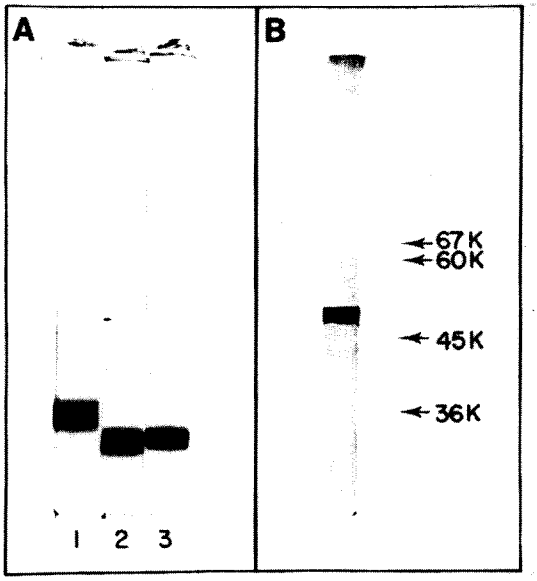

Fig.1. Polyacrylamide gel electrophoresis. (A) SDS gel electrophoresis in 10% gels, 1 mM $CaCl_2$ (pH 8.9) of: (1) rat testicular calmodulin; (2) rat hepatoma calcium binding protein; (3) rat skeletal muscle parvalbumin. The gels were overloaded with 50 μg each protein to visualise possible impurities. (B) SDS gel electrophoresis in 7.5% gels of 20 μg calmodulin-affinity purified phosphodiesterase. Arrows indicate the migration of albumin (M_r 67 000), catalase subunit (M_r 60 000), ovalbumin (M_r 43 000), and lactate dehydrogenase subunit (M_r 36 000).

Table 1
Estimate of calmodulin contamination during purification of tumour calcium-binding protein

Fraction	Vol. (ml)	^{125}I (cpm/ml)	CaM[a] (mg)	TCaBP[b] (mg)
100 000 × *g* supernatant	850	5×10^8 (100%)	7.2 (100%)	28.1 (100%)
pH 4 [65% $(NH_4)_2SO_4$]	125	1×10^8 (20%)	1.6 (22%)	20.4 (73%)
DEAE-cellulose	1.5	1×10^6 (0.2%)	n.d.	15.1 (54%)
Sephadex G-50	12.5	5×10^5 (0.09%)	n.d.	9.2[c] (33%)

[a]Measured by radioimmunoassay [14]; [b]measured by rocket immunoelectrophoresis [3]; [c]measured as dry weight after lyophilisation; n.d., not detectable

Extract from 350 g tumour was spiked with 5 μg ^{125}I-labeled calmodulin

Sephadex G-50, and CNBr-activated Sepharose were from Pharmacia (Dorval, P. Q.). Affigel-601, Chelex-100 and PAGE materials were from BioRad. Trifluoperazine was the gift of Smith, Kline and French (Toronto, Ont.). Thin-layer cellulose sheets were from Brinkmann (Rexdale, Ont.). All other chemicals were of the highest purity available.

CaM–Sepharose (3 mg CaM/g gel) was prepared as in [13], and CaM was iodinated (140 000 cpm/ng) using the [^{125}I]Bolton-Hunter reagent [14]. [CaM] was measured by radioimmunoassay using an antibody raised in this laboratory in sheep. The anti-CaM IgG was purified on CaM–Sepharose [15]. Peptide maps of the tumour CaBP or CaM were produced by treating 1 mg samples of performic acid-oxidised proteins with TPCK-treated trypsin [16,17]. The pep-

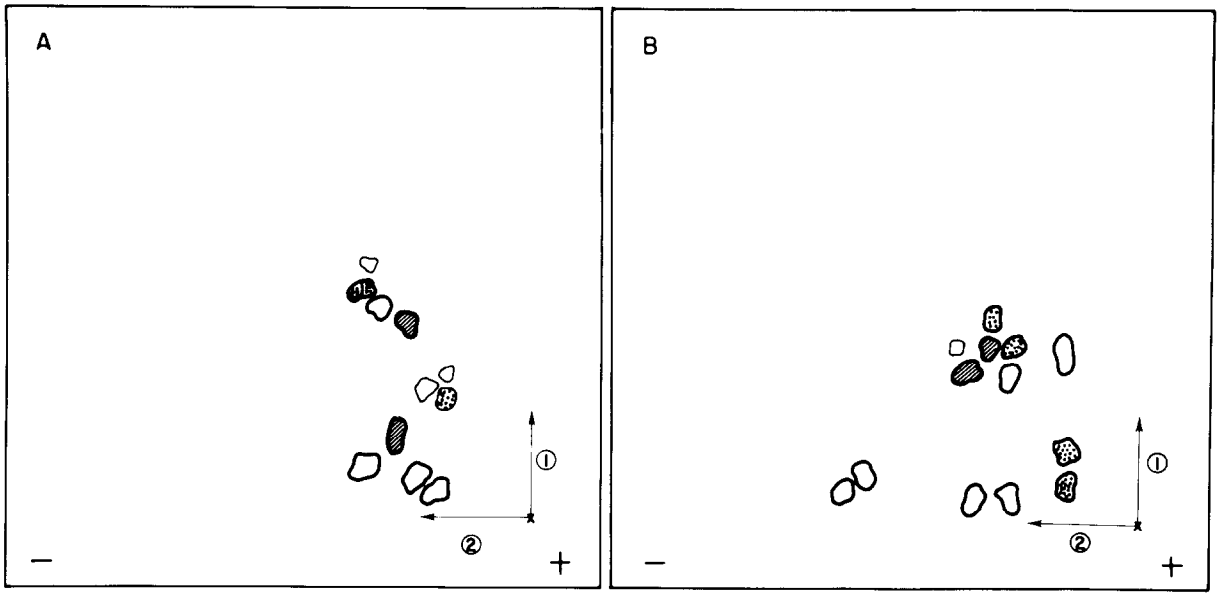

Fig.2. Peptide map of (A) tumour calcium-binding protein and (B) calmodulin. Samples (1 mg) of performic acid-oxidised [16] proteins, which had been exhaustively dialysed against 50 mM NH_4HCO_3, 1 mM EGTA (pH 8.0) were hydrolysed with TPCK-treated trypsin. Samples (50 μg) were chromatographed on cellulose thin-layers in *n*-butanol:pyridine:acetic acid:H_2O (50:33:1:40), followed by electrophoresis (11 mA/100 min) in the second dimension in pyridine:acetic acid:H_2O (1:10:89) [16,18]. The peptides were stained with Cd-ninhydrin [33], and tyrosine located by spraying with α-nitroso-β-naphtol [34]. Thick contours were clearly visible spots, thin contours were faintly visible, hatched areas were yellow spots, and stippled areas stained for tyrosine.

tides were separated in two dimensions on cellulose plates [18].

Cyclic GMP phosphodiesterase was purified from rat heart by two DEAE–Sephacel steps following the procedure for bovine heart [19]. This enzyme was then further purified by CaM affinity chromatography [13] yielding a major protein band of M_r 53 000 (fig.1; [13,19]). Enzyme activity was measured by direct assay using boronate affinity methods [20].

All reagent solutions had <2 μM calcium by atomic absorption spectrometry (Pye Unicam SP 191) when finally used. Components were treated with Chelex-100 ion-exchange resin to reduce the [calcium] to this level. Free [calcium] was calculated using the computer programme in [21]. Plastic laboratory ware was used for all enzyme studies.

3. Results and discussion

Before investigating the potential of the tumour CaBP to activate a CaM-dependent enzyme, it was first necessary to show that the purified protein was free of CaM contamination. The high speed supernatant of a Morris hepatoma 5123 homogenate [2] was spiked with 5 μg ^{125}I-labeled CaM. The tumour CaBP was purified [2], and each step assayed for CaM and ^{125}I-radioactivity (table 1). The final material from Sephadex G-50 had no radioimmunoassayable CaM, and the ^{125}I content equated to a maximum possible contamination of the 9.2 mg tumour CaBP with 0.07% CaM by weight. This preparation of the tumour CaBP was considered essentially free of CaM, and was used throughout these studies.

Besides showing the tumour CaBP to be free of CaM contamination, it was also necessary to show that the tumour protein was not a fragment of CaM before enzyme studies were undertaken. Tryptic peptide maps of both rat calcium-binding proteins were produced. CaM yielded 13 peptides and the tumour CaBP yielded 11 (fig.2). Differential staining with Cd-ninhydrin, and the location of peptides containing Tyr showed that all 11 peptides of the tumour CaBP were clearly different from the CaM peptides. This supported the suggestion that the tumour CaBP could not be a fragment of CaM [2]. When a mixture of the hydrolysates from the two proteins was mapped the entire 24 peptides could be distinguished (not shown).

Since the tumour CaBP was neither contaminated with, nor a fragment of, CaM, its effect on cyclic nucleotide phosphodiesterase was studied. The CaM affinity purified enzyme was, as expected, stimulated by CaM (fig.3A). Chelation of calcium in the reaction mixture with EGTA eliminated this stimulation. The phosphodiesterase was also stimulated in a calcium-dependent manner by the smaller tumour CaBP (fig.3A). However ~10-fold more tumour protein

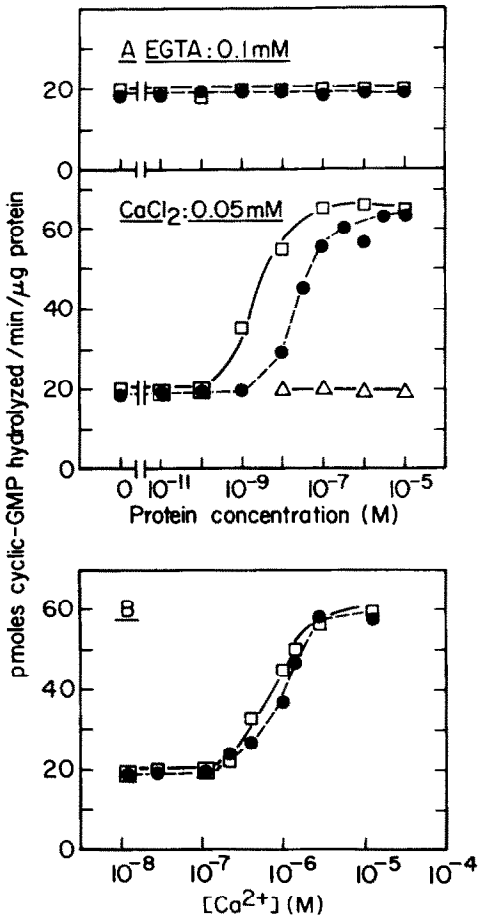

Fig.3. The stimulation of calmodulin-dependent cyclic GMP phosphodiesterase by tumour calcium-binding protein. The assay was performed in 200 μl final vol. containing 100 mM KCl, 50 mM $MgCl_2$, 50 mM Hepes, 0.2 mM dithiothreitol, 0.2 mg/ml bovine serum albumin (pH 7.0) and 0.5–5 μM cyclic [^{3}H]GMP (1000–200 cpm/mol). Reaction conditions at 37°C were adjusted so that <15% of the substrate was hydrolysed. The enzyme (40 ng protein) was incubated for 5 min (1 μM cyclic GMP) under conditions: (A) of changing calcium-binding protein concentration; (B) of changing free calcium concentration (1 mM EGTA) at 1 μM calcium-binding protein. (□) Calmodulin; (•) tumour calcium-binding protein; (△) parvalbumin. All points are the mean of ⩾10 separate determinations. The SEM was too small to be shown.

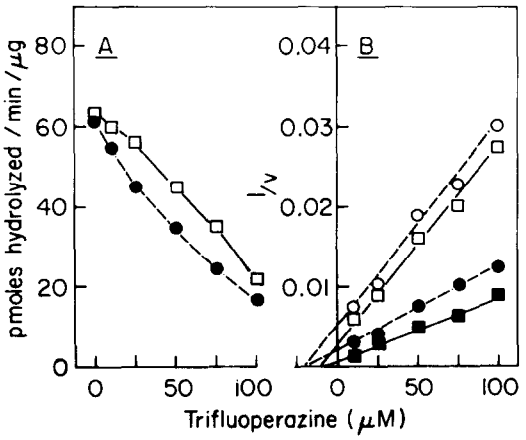

Fig.4. Inhibition of cyclic GMP phosphodiesterase stimulation by trifluoperazine. Assay conditions were as in fig.3. (A) decreased hydrolysis of cyclic GMP (1 μM) with increased concentration of trifluoperazine. (B) Dixon plot [35] of enzyme activity at 0.5 μM and 5 μM cyclic GMP. (□,■) Calmodulin; (○,●) tumour calcium-binding protein.

was required to give the same stimulation as CaM. This could not have been due to 10% contamination of the tumour protein with CaM (table 1). Parvalbumin, of similar size and calcium binding capacity as the tumour CaBP was unable to activate the phosphodiesterase even up to 10 μM confirming [4,11]. The free calcium requirement for stimulation of cyclic GMP hydrolysis was similar for both CaM and the tumour CaBP (fig.3B).

Trifluoperazine, an antipsychotic agent, binds to CaM [22], and inhibits CaM-dependent enzymes [5,23,24]. The drug inhibited cyclic nucleotide hydrolysis whether it was stimulated by CaM or the tumour CaBP (fig.4A). However the K_i for the non-competitive inhibition by trifluoperazine with the tumour protein (20 μM) was double that with CaM (fig.4B).

Thus a M_r 11 500 calcium-binding protein from neoplastic liver has the potential to activate a CaM-dependent enzyme from normal rat tissue. This protein cannot be detected in normal tissue [1,3]. Another example of a change in normal levels of a calcium-binding protein in tumour cells may be the increase in CaM described in [25–29]. CaM can stimulate DNA synthesis in quiescent non-neoplastic rat liver cells in vitro [30], and the tumour CaBP can also mimic this action of CaM (A. L. Boynton and J. F. Whitfield, unpublished). While the function of the tumour CaM-like protein is unknown, its presence may be linked, along with abnormal CaM levels, to perturbed calcium control mechanism, vividly demonstrated by the ability of neoplastic cells to grown in abnormally low extracellular calcium [31,32].

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