AN INCREASE IN CALMODULIN DURING GROWTH OF NORMAL AND CANCEROUS LIVER IN VIVO

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1. Introduction

The calcium-dependent aspects of such diverse processes as contraction, secretion and nerve function seem to be mediated by the ubiquitous calcium receptor protein, calmodulin (CaM) [1]. Another well-established, calcium-dependent process is cell proliferation both in vivo and in vitro [2]. Whether CaM is involved with this action of calcium remains unclear. However several lines of evidence suggest that this calcium receptor protein does have some function in the control of cell proliferation:

(i) An increase in CaM has been found in a tumour cell line as the cells synchronously enter the DNA synthetic phase of the cell cycle [3];
(ii) CaM does have the ability to stimulate DNA synthesis in isolated liver cells [4];
(iii) Many cell lines when transformed by tumour viruses have increased amounts of CaM [5–7].

This report submits evidence for the first time of a change in CaM during growth of normal liver, regenerating in vivo, and a greater content of CaM in many solid tumours of liver.

2. Materials and methods

Partial hepatectomy (68%) was performed between 7:0 and 10:0 pm [8] on specific-pathogen free, male, Sprague-Dawley rats (190–230 g). Fetal and neonatal livers were also from Sprague-Dawley stock.

Morris hepatomas 5123tc, 5123tch, 7288tc and 7795 were maintained in this laboratory as in [9] using Buffalo rats (Microbiological Assoc., Walkersville MD). The other Morris hepatomas were supplied from Howard University (Washington DC). The Yoshida, Novikoff and LC-18 hepatomas were from the Mason Research Institute Tumour Bank (Worcester MA).

Tissues to be assayed for CaM were excised and immediately homogenised (1:10, w/v) in the ice-cold isolation medium (150 mM NaCl, 10 mM $MgCl_2$, 10 mM imidazole, 1 mM EGTA, 1 mM mercaptoethanol, pH 7.5). After a sample of the total homogenate was removed for total CaM assay, the remainder was centrifuged at 105 000 × *g* for 30 min in a Beckman L265B centrifuge. The particulate pellet was washed, recentrifuged and diluted to the original volume with isolation medium. Total, soluble and particulate samples were heated for 5 min to 80°C, rapidly cooled, sonicated, and re-centrifuged at 105 000 × *g*. The resulting extracts were stored at −80°C to await assay.

The radioimmunoassay for CaM was based on procedures in [10,11]. Rat testicular CaM was purified to homogeneity [12], and an antiserum raised in sheep using performic acid oxidised material [13]. The sheep were bled after a series of CaM inoculations consisting of 5 mg on day 0 and 2 mg on days 11 and 24. The crude immunoglobulins [14] were passed through a column of DEAE-Affigel blue (BioRad, Mississauga, Ont.). The material not binding to the column was dialysed against 100 mM boric acid, 25 mM sodium borate, 75 mM NaCl (pH 8.4). The anti-CaM immunoglobulin in this fraction was absorbed by antigen affinity chromatography on CaM–Sepharose [15] and eluted with 100 mM glycine (pH 2.7) [10]. The eluate was neutralised immediately and dialysed against 100 mM NaCl, 10 mM imidazole (pH 7.4).

CaM was iodinated with the ^{125}I-labeled Bolton-

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Hunter reagent (Amersham, Oakville, Ont.) which yielded 120 000 cpm/ng CaM. The radioimmunoassay was performed in a final volume of 500 μl RIA buffer (75 mM NaCl, 1 mM EGTA, 125 mM Tris, 20 μg/ml bovine serum albumin (pH 8.4)) containing 40 000 cpm ^{125}I-labeled CaM, 2 μg anti-CaM, and standard amounts of CaM, or tissue extracts. The tubes were incubated at room temperature for 2 h, and then at 4°C for 20 h before precipitation of the immune complex with 1 ml Pluronic F-38 (BASF, Rexdale, Ont.) at a final concentration of 14% (w/v) following centrifugation at 6000 × *g* for 30 min. The precipitates were counted in a Beckman Biogamma counter.

A typical standard curve is illustrated in fig.1A. An excess of rat parvalbumin, or bovine lactalbumin did not displace CaM from the antibody. However, rabbit skeletal muscle troponin C did show some cross-reactivity at 5000 ng/tube (fig.1A). Such an interaction of troponin C with anti-CaM antisera was reported in [11,13]. Also illustrated in fig.1A is the ability of serially diluted liver extract to cross-react in a parallel fashion with the anti-CaM antisera. Such a parallel displacement indicated the presence in the liver extract of CaM or an immunologically very similar material [11,13].

CaM content was also measured by stimulation of a cyclic-nucleotide phosphodiesterase [1]. CaM deficient phosphodiesterase was purified from rat heart and assayed as in [16]. The extent of stimulation of the enzyme by known amounts of CaM is shown in fig.1B.

DNA synthesis was measured autoradiographically in rats injected with [^{3}H]TdR [17].

3. Results and discussion

The CaM content of either the soluble or particulate fraction of liver did not change after sham hepatectomy. However when 68% partial hepatectomy was performed, the CaM content of the soluble fraction of the liver remnant increased between 6–8 h following surgery, and reached a peak concentration which was 3-fold higher than control (fig.2A). The

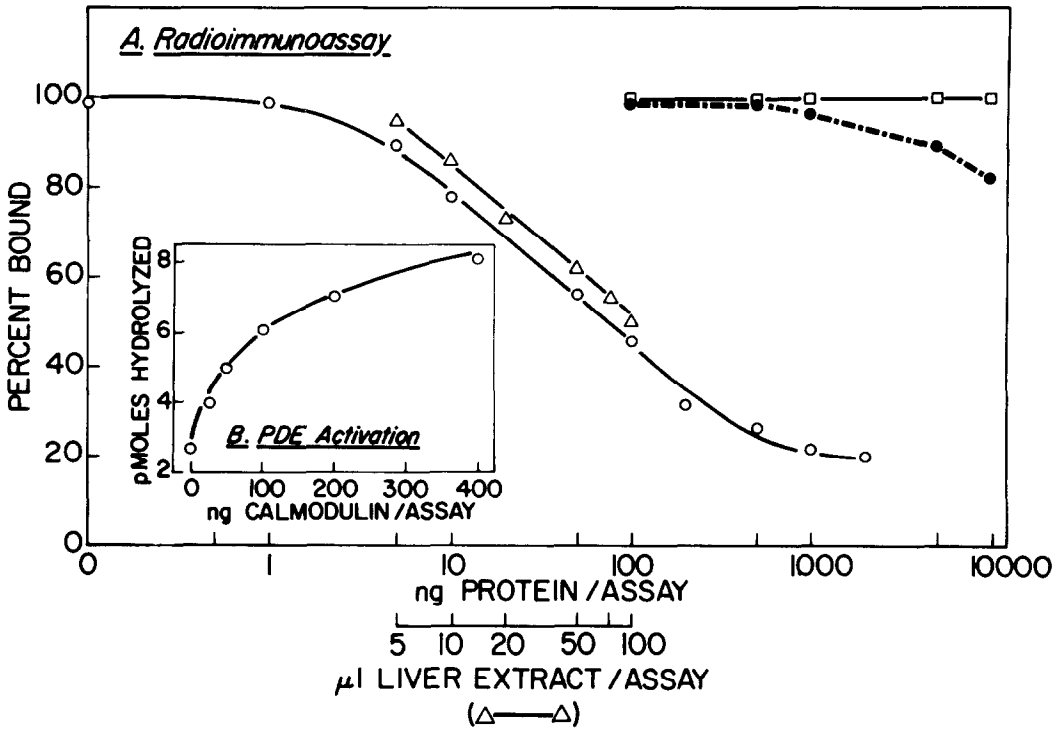

Fig.1. The measurement of calmodulin content by (A) radioimmunoassay and (B) PDE activation: (A) (○——○) CaM; (□——□) parvalbumin or lactalbumin; (●– – –●) troponin C. Samples of tissue extracts were assayed in triplicate at at least two different dilutions. (B) (○——○) CaM. Tissue extracts were assayed in duplicate at at least 3 different dilutions.

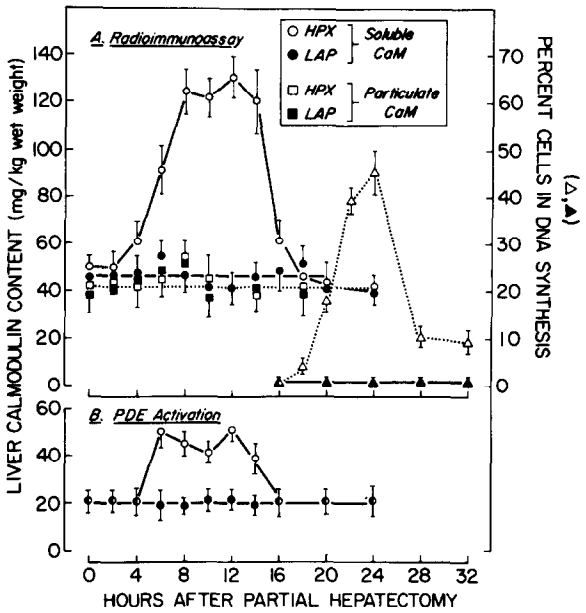

Fig.2. Changes in the particulate and soluble calmodulin contents of regenerating liver as determined by radioimmunoassay and PDE activation. Values are the mean ± SEM of between 4–12 separate animals.

CaM content then returned to normal by 18 h, which was shortly before the synthesis of DNA began (fig.2A). No change occurred in the particulate fraction of regenerating liver (fig.2A).

Although the radioimmunoassay was considered specific for CaM (see section 2), other proteins have been reported that cross-react with anti-CaM antisera [18,19]. Therefore the soluble fractions of regenerating liver were also tested for their ability to activate CaM-dependent cyclic nucleotide phosphodiesterase. This assay confirmed that CaM increased during liver regeneration (fig.2B), although the absolute amounts of CaM were lower than obtained with radioimmunoassay. This underestimate by the phosphodiesterase activation assay has been observed by others [11].

The time when CaM increased during liver regeneration (fig.2) coincides with the time when calcium is required for the initiation of DNA synthesis [17]. A similar late prereplicative requirement for calcium has been demonstrated for liver cells in vitro [20], which also coincides with an increase in cellular CaM [21]. The relevance of the CaM rise to the calcium dependent prereplicative steps remains to be established.

Besides regenerating liver, the CaM content of other in vivo growing tissues of liver origin was measured. Fetal or neonatal liver had the same CaM concentration as adult liver. However liver tumours, induced by various means, and of different growth rates all contained more CaM than normal adult, or fetal liver as shown by both radioimmunoassay and PDE assay (table 1).

While this work was in progress, a report appeared of an increased CaM content (determined by PDE activation) of one Morris hepatoma [22]. It had been suggested that although the total CaM content of some Morris hepatomas was normal, a redistribution occurred causing an increase in the soluble CaM in the tumours [23]. These suggestions, plus the extensive

Table 1
Calmodulin content of various solid tumours of rat liver origin

Tissue	Total CaM (mg/kg)		Tumour growth rate[a]
	RIA assay	PDE assay	
Liver			
Adult[b]	78 ± 5 (20)	40	–
Neonatal, 2 day	80 ± 7 (3)	40	–
Fetal, 8 day	71 ± 9 (6)	40	–
Hepatoma			
Morris 7288Ctc	217 ± 27 (12)	60	2
5123tcg	220 ± 31 (8)	–	2–3
5123tc	200 ± 85 (20)	125	3–4
3683Ftc	207 ± 19 (6)	200	4
8994	361 ± 44 (6)	200	4
7288C	186 ± 15 (6)	60	4
8995	275 ± 23 (6)	200	6
28A	170 ± 19 (6)	100	8
5123C	201 ± 16 (6)	–	8–9
7800	104 ± 9 (6)	80	10
7794A	214 ± 27 (6)	50	12
7795	100 ± 16 (6)	–	16
21	161 ± 28 (6)	200	22
7787	228 ± 24 (6)	150	30
9618A	202 ± 13 (6)	100	48
Yoshida[c]	212 ± 32 (6)	–	–
Novikoff	126 ± 17 (6)	100	–
LC-18	357 ± 49 (6)	150	–

[a] Time in weeks between transfers averaged over 10 generations (time in weeks from implantation to the point at which tumour extension requires that the host be killed)
[b] Buffalo or Sprague-Dawley strain; [c] Carried in host as ascites

Values are mean ± SEM (no. separate estimates); PDE assay values are the mean of 3 separate estimates

data in table 1, combine to demonstrate that the increase in CaM content seen in virally transformed cells in vitro [5–7] may be a general attribute of the neoplastic state. Indeed the evidence of an increase in CaM in growing normal liver during regeneration suggests that CaM may somehow be involved with the control of cell proliferation. The potential of CaM as a growth modulator has been already demonstrated by its ability to stimulate DNA synthesis of liver cells in vitro [4].

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