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Increased calmodulin synthesis in the pre-replicative phase of rat liver regeneration

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A cytosolic calmodulin wave has been described in the pre-replicative phase of rat liver regeneration. Here we demonstrate that this calmodulin increase is reduced by injection of cycloheximide (translation inhibitor) and actinomycin D (transcription inhibitor). In addition, we found that calmodulin mRNA levels are increased during the early pre-replicative period of liver regeneration, and this increase is affected by actinomycin D. Together, these results strongly indicate that the calmodulin wave observed in the pre-replicative phase of liver regeneration is due to de novo synthesis of this protein.

Ca^{2+}-binding protein; Calmodulin; Liver regeneration

1. INTRODUCTION

Calcium together with calmodulin is important for the initiation of DNA synthesis in normal and neoplastic mammalian cells [1–3]. Elevated calmodulin levels have been described in some cell lines virally transformed in vitro [4,5]. Elevated calmodulin levels at the G_1-S boundary and a functional calmodulin gene are required for the cell-cycle progression [6,7]. The proliferation of cultured cells is prevented or delayed by anti-calmodulin drugs [3,6].

Partial hepatectomy activates the proliferation of hepatocytes of the remaining liver, and DNA synthesis starts about 18 h after surgery. Previous studies suggested that cytosolic calcium controls liver regeneration [1,8]. In addition, a calmodulin wave in liver regeneration has been observed between 6 and 14 h after surgery, with maximum levels between 8 and 12 h after operation [9]. Delay of calmodulin wave and DNA synthesis by an anticalmodulin drug (trifluoperazine) indicates that both events may be related in liver regeneration [10].

So far, the origin of the calmodulin wave in the pre-replicative phase of liver regeneration has remained unclear. It has been suggested that the increase in cytosolic calmodulin during liver regeneration may be due to an increased synthesis of the protein. Another possibility would be translocation of calmodulin from one cellular compartment to another (for example plasma membrane to cytosol) but it has been reported that calmodulin levels remain constant in the particulate fraction during liver regeneration [9]. A third possibility would be an altered calmodulin turnover.

Our results strongly suggest that the cytosolic calmodulin wave observed after partial hepatectomy arises from de novo synthesis of the protein.

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Abbreviation: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

2.1. *Animals*

Male Sprague-Dawley rats (7–8 weeks old) were used in all experiments. Animals were kept in a 12 h light-dark schedule with food and water provided ad libitum. Before surgery, performed under Ketolar® anesthesia, animals were fasted for 12 h. Sham operations were performed under identical conditions. Rats were killed by decapitation. Actinomycin D (Sigma, 1 μg/g body wt) or cycloheximide (0.66 μg/g body wt) were injected into the tail vein, 1 or 3 h after surgery.

2.2. *Tissue extracts*

Liver tissues were homogenized in 10 vols of 125 mM borate, 1 mM EGTA, 75 mM NaCl, pH 7.8 (Dounce homogenizer). After centrifugation (100000 × *g*), supernatants were heated (5 min, 95°C), cooled and re-centrifuged (50000 × *g*). Aliquots were frozen at −20°C until use.

2.3. *Protein and calmodulin measurements*

Protein concentrations were measured according to Lowry et al. [11] and Sedmak and Grossberg [12]. Calmodulin concentrations were determined by phosphodiesterase activation [13].

2.4. *2D-PAGE*

Purified proteins and extracts were labeled in vitro by reductive methylation [14] using [^{14}C]formaldehyde (NEN, specific activity 52 Ci/mol), separated by 2D-PAGE according to O'Farrell [15] and visualized by fluorography [16]. For isoelectric focusing, 20% Ampholine (LKB) pH 2.5–4 and 80% Ampholine (LKB) pH 3.5–10 (linear gradient 4–7.5) were applied.

2.5. *RNA isolation*

Total RNA was obtained as described by Chirgwin et al. [17]. Poly(A$^+$) RNA was prepared by affinity chromatography from total RNA according to Aviv and Leder [18].

2.6. *RNA blot*

Total RNA (25 μg) or poly(A$^+$) RNA (10 μg) were electrophoresed on gels containing 1.4% agarose and 6% formaldehyde and blotted onto Biodyne membrane (Pall, Glen Cove, NY) as described by Thomas [19].

2.7. *RNA dot blot*

Total or poly(A$^+$) RNA were processed as described by Berchtold and Means [20] according to Schloss et al. [21]. Nylon membranes (Biodyne, Pall Corp., Glen Cove, NY, USA) were hybridized and washed at high stringency as described in the manufacturer's manual. Kodak and Fuji X-ray film were exposed at −70°C and the signals were quantitated by measuring the absorbance of the film at 405 nm in a Virion Reader.

2.8. *Labeling of cDNA probes*

The following cDNA probes were used for hybridization: calmodulin cDNA [22], β-actin cDNA [23] and parvalbumin cDNA [20]. Labeling was performed according to Feinberg and Vogelstein [24] resulting in a specific radioactivity of 2–3 × 10^9 dpm/μg, and separated from free radioactivity by passage over a 1 ml Sephadex G50 column in 20 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, 0.25% SDS (pH 7.5).

3. RESULTS AND DISCUSSION

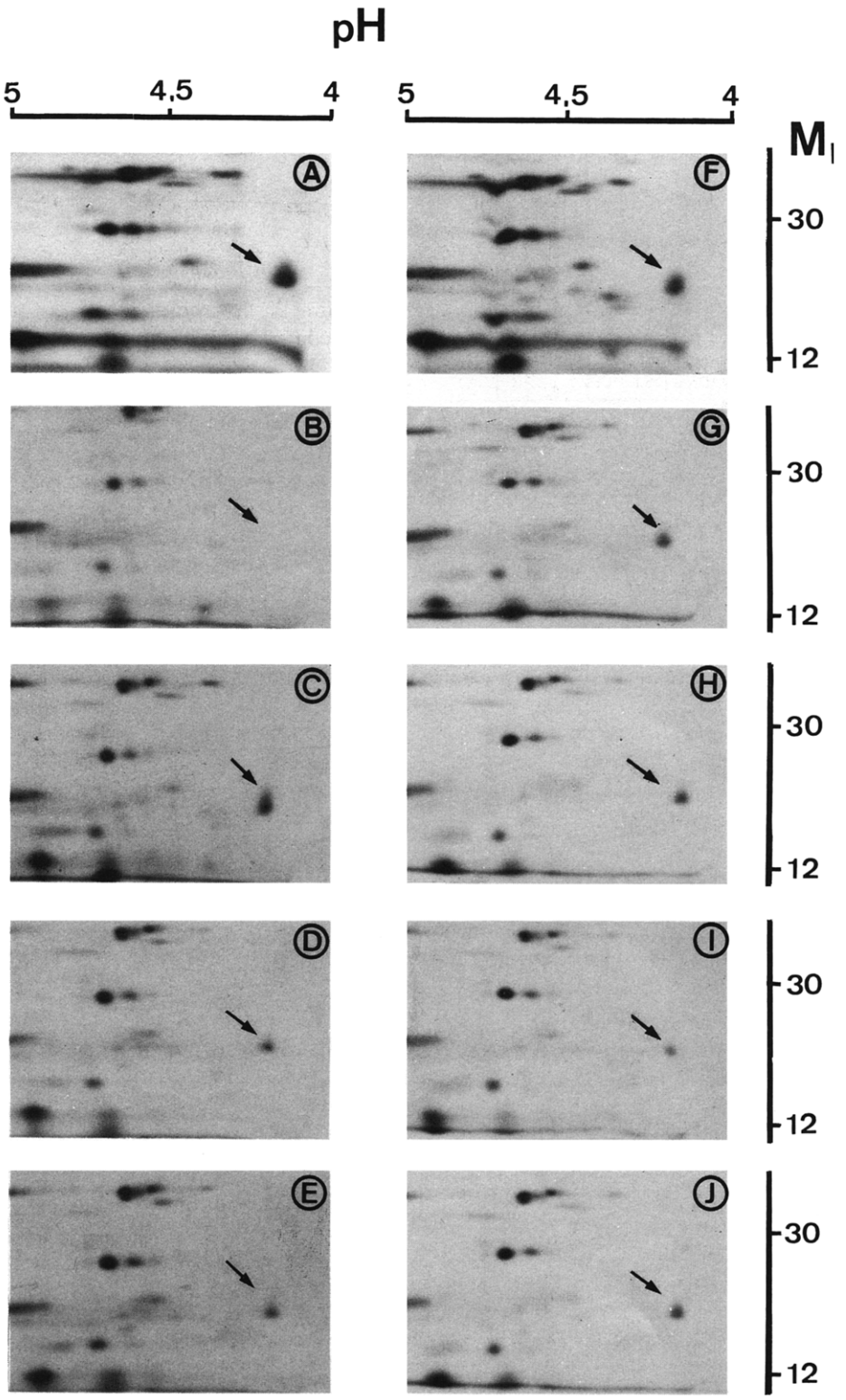
3.1. *Effect of protein synthesis inhibitors on calmodulin levels*

Cycloheximide and actinomycin D were injected into hepatectomized rats and the appearance of calmodulin in the cytosolic fraction at different time points of the pre-replicative phase of liver regeneration was followed by 2D-PAGE. Calmodulin (fig.1, arrows) is characterized by a molecular mass of 18 kDa and an isoelectric point of approx. pH 4.1. Calmodulin spots in regenerating livers of rats injected with cycloheximide (fig.1B,G,D,I) or actinomycin D (fig.1C,H,E,J) are weaker than the ones observed in livers of hepatectomized (fig.1A,F) or laparotomized rats (not shown).

Two general observations were made: (a) the calmodulin spot was weaker when cycloheximide was injected instead of actinomycin; (b) inhibitors injected 1 h after surgery caused a greater reduction in the calmodulin spot than those injected 3 h after surgery. Strongest inhibition was observed when cycloheximide was injected 1 h after hepatectomy, and in some samples the calmodulin spot was not even detectable (fig.1B).

The electrophoretic analysis was confirmed by quantitative measurement of cytosolic calmodulin by the phosphodiesterase activation method (fig.2). Calmodulin levels in regenerating livers of rats killed 8 and 12 h after partial hepatectomy are about twice that of control livers (mean value of non operated and laparotomized rats). When hepatectomized rats were injected with actinomycin D, a non-significant diminution of calmodulin levels was observed, but when cycloheximide was injected, calmodulin levels were in the range of control levels, or even lower, as in the case of some

Fig.1. 2D-PAGE of hepatic cytosol from hepatectomized rats: (A–E) killed 8 h after surgery and (F–J) killed 12 h after surgery. (A,F) No inhibitor injected; (B,G) injected with cycloheximide 1 h after surgery; (C,H) injected with actinomycin D 1 h after surgery; (D,I) injected with cycloheximide 3 h after surgery; (E,J) injected with actinomycin D 3 h after surgery.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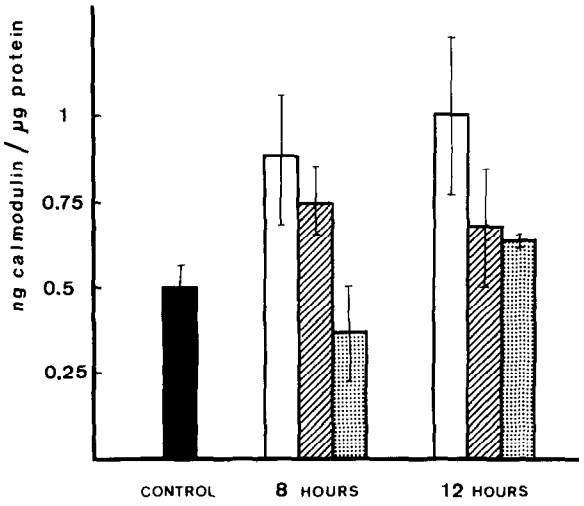

Fig.2. Calmodulin measurement in hepatic cytosol by the phosphodiesterase method. Control livers (■); regenerating livers (□); regenerating livers of rats injected with actinomycin D (▨); regenerating livers of rats injected with cycloheximide (▦).

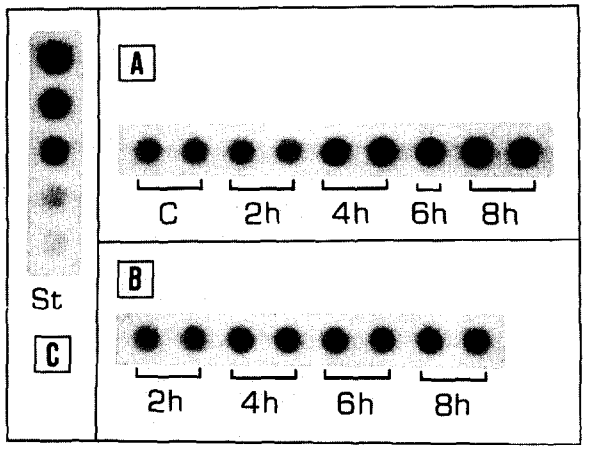

Fig.3. Changes in calmodulin mRNA assessed by dot blot hybridization of 10 μg of poly(A^+) RNA. (A) Calmodulin cDNA hybridization of samples from control and hepatectomized rats. (B) Calmodulin cDNA hybridization of samples from hepatectomized rats injected with actinomycin D (150 μg/150 g body wt) 1 h after surgery. (C) Calmodulin cDNA hybridization of rat testis poly(A^+) RNA, used as standard.

rats killed 8 h after surgery. This is in agreement with the 2D-PAGE analysis.

3.2. *Calmodulin mRNA measurement*

This study was performed from 0 to 8 h after surgery since the calmodulin wave has its maximum at 8–12 h after hepatectomy.

Calmodulin mRNA levels are not markedly changed 2 h after partial hepatectomy, but at 4, 6, and 8 h after surgery there is an increase of calmodulin mRNA, at 8 h being 151% of the value found in control livers (table 1). Measurement of β-actin mRNA on the same Biodyne paper was used as a positive control since it has been described by Friedman et al. [25] that β-actin increases during liver regeneration. 8 h after surgery we measured a value of 191% compared to controls. Parvalbumin was chosen as a control for background signals since it is not expressed in liver. Parvalbumin cDNA hybridization gave no signal in either control or regenerating livers. Calmodulin mRNA was also measured on poly(A^+) RNA dot blots. Two groups of rats were used for this experiment: (i) hepatectomized rats killed 2, 4, 6, and 8 h

Table 1

Increment of calmodulin and β-actin mRNA measured in units of densitometric absorbance/20 μg total RNA

Time after surgery (h)	Calmodulin	%	β-Actin	%
0 (Control)	800.2 ± 16.6	100	669.0 ± 88	100
2	776.2 ± 28.1	86	739.6 ± 49	111
4	824.2 ± 12.8	114	991.0 ± 79	148
6	854.2 ± 3.8	131	1296.2 ± 57	194
8	890.5 ± 15.3	151	1278.7 ± 372	191

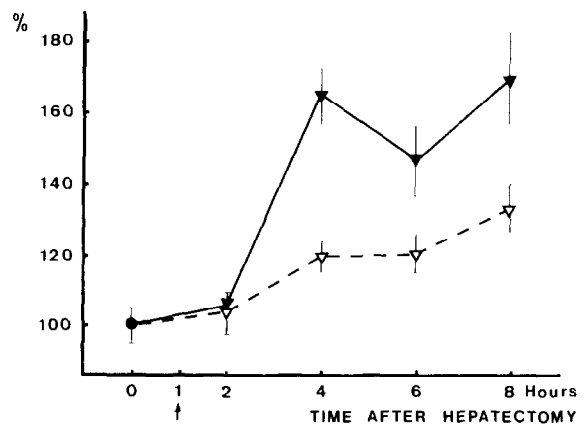

Fig.4. Calmodulin messenger RNA increase. (▲—▲) Hepatectomized rats; (Δ---Δ) hepatectomized rats injected with actinomycin D 1 h after surgery.

after surgery, and (ii) hepatectomized rats which were injected with actinomycin D and then killed at the same time intervals in the first group. Signals obtained from the first group were higher than the ones from the second group (fig.3). The first group shows a clear increase of calmodulin mRNA of 171% with respect to the control value, but hepatectomized rats injected with actinomycin D exhibit a short and gradual increase of calmodulin mRNA reaching a maximum value of 134%.

When protein synthesis was inhibited by cycloheximide, the levels of calmodulin dropped to normal (mean value of non-operated and laparotomized rats), indicating that the calmodulin wave is due to new translation of calmodulin.

However, the effects of actinomycin D were more complex. Although calmodulin mRNA levels were strongly reduced in livers of hepatectomized rats treated with actinomycin D, calmodulin protein levels did not change significantly. This discrepancy may be explained as follows: as shown in fig.4, hepatectomized rats injected with actinomycin D exhibit a 30% increase of calmodulin mRNA levels compared to the control. It is possible that this slight increase in mRNA is still sufficient to produce similar amounts of calmodulin in treated regenerating livers when translation is not inhibited. The 30% increase in calmodulin mRNA could be because (i) the dose of actinomycin D was not sufficient to completely block transcription, or (ii) there is an altered turnover rate of calmodulin mRNA in regenerating livers.

Partial hepatectomy leads to the activation of a large number of cells, which subsequently enter the G_1 phase of the cell cycle. The calmodulin wave previously described to take place in the prereplicative phase of liver regeneration [9,10], finishes at 16 h after surgery, i.e., 2 h prior to the beginning of DNA synthesis. It is probable that the role of calmodulin is related with the progression through the cell cycle as has been suggested in a previous report using C127 mouse cells transformed by a BPV vector containing the calmodulin gene [26]: elevated calmodulin levels affect the rate of cell proliferation by increasing the rate at which cells progress through G_1.

Our results demonstrate that the calmodulin wave in liver regeneration is due to an elevated protein production; translation as well as transcription are both increased. This is in agreement with observations from in vitro experiments, demonstrating new synthesis of calmodulin when quiescent cultured cells (CHO-K1) re-enter in the cell cycle [27].

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