CALMODULIN ACTIVITY DURING THE CELL-CYCLE OF TETRAHYMENA

Dissertation submitted to the Jawaharlal Nehru University
in partial fulfilment of the requirements for
the Degree of
MASTER OF PHILOSOPHY
1984

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C E R T I F I C A T E

This is to certify that the research work embodied in this dissertation has been carried out in School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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ACKNOWLEDGEMENT

I express my deep sense of gratitude to Dr. Samar Chatterjee, my supervisor, for rendering guidance and assistance in the completion of this assignment.

I am equally grateful to Prof. Asis Datta, Dean, School of Life Sciences and Prof. P.C.Kesavan, former Dean, for providing all the facilities for research.

I owe a special debt of gratitude to Dr. S.K. Sopory, Associate Professor, School of Life Sciences, JNU, for his expertise and experienced discussions which enabled us to solve the experimental problems during our study.

My grateful thanks are due to Dr. C.S.K.Mayanil, Miss Rina Das, Mr K.Natarajan, Dr. N.Menon, Mr. Kazmi, Mr. N.K.Dash, Mr. Karunakar Rao and Mr. A. Verma for their valuable help and suggestions. I am also thankful to Mr Alexandar and Mr R.N.Saini for their expert technical assistance.

Thanks are due to Jawaharlal Nehru University for providing me financial help in the form of J.R.F.

(JITENDRA SINGH)

ABBREVIATIONS USED

BSA	: Bovine Serum Albumin
$CaCl_2.2H_2O$	: Calcium chloride dihydrate
$CrK(SO_4)_2.12H_2O$	: Chrome alum
EDTA	: Ethylenediamine tetra acetic acid
EGTA	: Ethylene glycerol-bis- β amino ethyl ether N, N, N', N' tetra acetic acid
^3H-TdR	: Tritiated thymidine
K_2HPO_4	: Dipottasium hydrogen phosphate
$K\ H_2\ PO_4$	: Pottasium dihydrogen phosphate
$Mg(CH_3COO)_2.4H_2O$	: Magnesium acetate
$MgSO_4.7H_2O$	: Magnesium sulfate
Na_2HPO_4	: Disodium hydrogen phosphate
$(NH_4)_2\ SO_4$	: Ammonium sulfate
PAGE	: Polyacrylamide gel electrophoresis
PMSF	: Phenyl methyl sulfonyl fluoride
POP	: 2,5 - Diphenyloxazole
POPOP	: 1,4-bis [2-(5.phenyloxazolyl)] benzene phenyl-oxazolyl phenyl
SDS	: Sodium Dodecyl sulfate
TCA	: Trichloro acetic acid
TEMED	: N, N, N', N' tetramethylethylene diamine
TES	: N-tris-(hydroxymethyl) methyl 2-amino ethanosulfonic acid
TFP	: Trifluoperazine
Tris	: Trihydroxy methyl aminoethane

INTRODUCTION

Before his death in 1959, L.V. Heilbrunn was a pioneering advocate of calcium as a key agent responsible for regulating many intercellular functions. This idea was somewhat ahead of its time, because the technique for manipulating low concentration of calcium had not yet been discovered. We, now, know that he was essentially correct in his views, although in his life time his proposals were considered somewhat extreme and controversial (Heilbrunn, 1956).

Calcium is required for the maintainance of optimal growth and functioning of living organisms. Ca^{2+} (ionic form of calcium) affects all aspects of cellular physiology and has been implicated in the regulation and modulation of various cell processes such as cell adhesion, motility, microtubule assembly, regulation of endocytic and exocytic secretion and initiation of DNA synthesis etc. The role of Ca^{2+} has been implicated in the mechanism of action of many neurotransmitters (both synthesis and release), digestion of food and adsorption of nutrients, formation and maintainance of bone and teeth. The regulation of blood clotting and wound healing, functioning of various enzymes and receptors, changes in metabolic states and

regulation of photochemical events in animals and plants are also mediated by Ca^{2+}(Val Eldik, 1982). Recently, the regulatory role of Ca^{2+} in cell division process has been reviewed (Charp and Whitson, 1982).

Agents or stimuli (i.e. hormones, potential changes etc.) that act on the membrane to increase its permeability to Ca^{2+} by this means enhance concentration of Ca^{2+} inside and stimulate the calcium sensitive reactions in the cell. Calcium, therefore, may be thought of as a coupling agent or messenger between stimuli acting on the cell membrane and processess that take place deep inside the cell (Ecker and Randall, 1978).

It has been recently shown that Ca^{2+} requires the binding with a group of low molecular weight proteins for its biological activity. Four different classes of calcium-binding proteins, which are involved in a variety of biological functions, have been implicated in the mechanism of how proteins are involved in the molecular basis of calcium action (Val Eldik, 1982). These are:

(I) The proteins containing gamma-carboxyglutamic acid provide an example of how a calcium binding protein is involved in interface reactions and lipidprotein interactions.

(II) Concanavalin A and calcium binding lectins are examples of how calcium is involved in protein: carbohydrate interactions and these lectins are very important for potentially mediating chronic effects of calcium in development and differentiation.

(III) Calcium-binding hydrolytic enzymes where calcium is directly involved in enzyme catalysis. Phospholipase A_2 is also another example of how calcium, proteins and lipids interact in mediating the biological effects of calcium.

(IV) Calcium modulating proteins like Calmodulin, Parvalbumin, a tissue specific protein (S-100) and a hormone induced protein (Vitamin D-dependent calcium binding protein) are best examples of how proteins mediate the acute, regulatory effects of calcium.

Among these calcium binding proteins, calmodulin is the ubiquitious regulatory Ca^{2+}-binding protein which has attracted wide attention of the investigators for its multi-faceted roles.

Calmodulin

Calmodulin (CaM) is the name proposed by Cheung in 1980 for a well-characterized protein which is now accepted as one of a group of several homologous

calcium-binding proteins such as troponin-C, parvalbumin and myosin light chains in most eukaryotic cells so far examined and has multiple calcium dependent activities (Klee et al., 1980; Vandermeers, 1980; Scharff, 1981; Cheung, 1982; Lin, 1982; Means et al., 1982; Levine and Dalgarno, 1983).

Discovery

CaM was first discovered as a heat stable dissociable activator in a crude cyclic nucleotide phosphodiesterase preparation from bovine brain that could be removed during purification by DEAE-cellulose chromatography by Cheung in 1970 and independently as a calcium dependent activator of phosphodiesterase by Kakiuchi and Yamuzaki in the same year. Later Teo and Wang in 1973 showed that the two proteins are identical.

Distribution

CaM is ubiquitiously distributed in eukaryotes. A wide range of different tissues from mammalian species such as cow, pig, hamster, mouse, rat, sheep, rabbit, human etc. and from non-mammal vertebrates such as xenopus, electric eel and chicken have been examined and are found to contain CaM. Inverterbrates such as earthworm, round worm, mealworm, clam, sponges, blue crab, sea anemone, sea-pansy, octopus, scallop,

sea-urchin, star fish and protozoa such as Tetrahymena pyriformis, Paramecium tetraurelia, Euglena glacilis and Amoeba proteus etc. Higher plants such as cotton seed, spinach leaves, peanuts and barley, Eumycophyta such as mushroom and Blastocladiella emeronii, Myxomycophyta (Dictyostelium discoideum) and Chlorophyta (Chlamydomonas) are also found to contain calmodulin. Among rat tissues, brain and testes are known to contain especially high amount of CaM; testes containing twice as much CaM as the brain. High concentrations of CaM from 1 to 50 μM have also been reported from a variety of mammalian tissue (See Means, 1982).

Physico-chemical properties of Calmodulin

Physical properties	Specific value
Molecular weight	16,700 (as determined from bovine brain and rat testes)
Heat resistence	$t_{\frac{1}{2}}$ = 7 min at 100°C
Isoelectric pH	3.9
Ca^{2+} - binding	4 mol/mol; K_d=2.4 μM
Conformation	40-50% alpha-helix
Amino acid composition	148 amino acids presence of trimethyllysine residue at position 115; absence of cysteine; absence of tryptophan resulting in a low extinction coefficient ($E^{1\%}_{280}$ of 2.0);

Physical properties	Specific value
	a high phenylalanine to tyrosine ratio resulting in an unusual absorption pattern of UV spectrum with 5 peaks at 251, 258, 264, 268 and 275 nm.
Sequence homology	50% trponin C
Drug binding	Phenothiazines, W compounds
Hydrophobicity	Increased by Ca^{2+}

CaM is an acidic protein remaining stable when subjected to heat treatment at either neutral or acidic pH. A solution containing CaM can be heated for 5 minutes at 90°C with no appreciable loss of biological activity as assessed by the ability to stimulate phosphodiesterase or myosine light-chain kinase. Although CaM is heat resistent it is not truly heat stable (See Means, 1982).

The protein is unique as it has no intrinsic enzymatic properties yet it regulates the activities of a diverse spectrum of important enzymes and serves as a major intracellular Ca^{2+} acceptor. The protein invariably lacks tissue and species specificity- another indication that it is a fundamental regulator (Means, 1980).

Structure

CaM contains four metal binding sites. Each of the four putative Ca^{2+}-binding domains is flanked by two stretches of alpha-helix. This structure

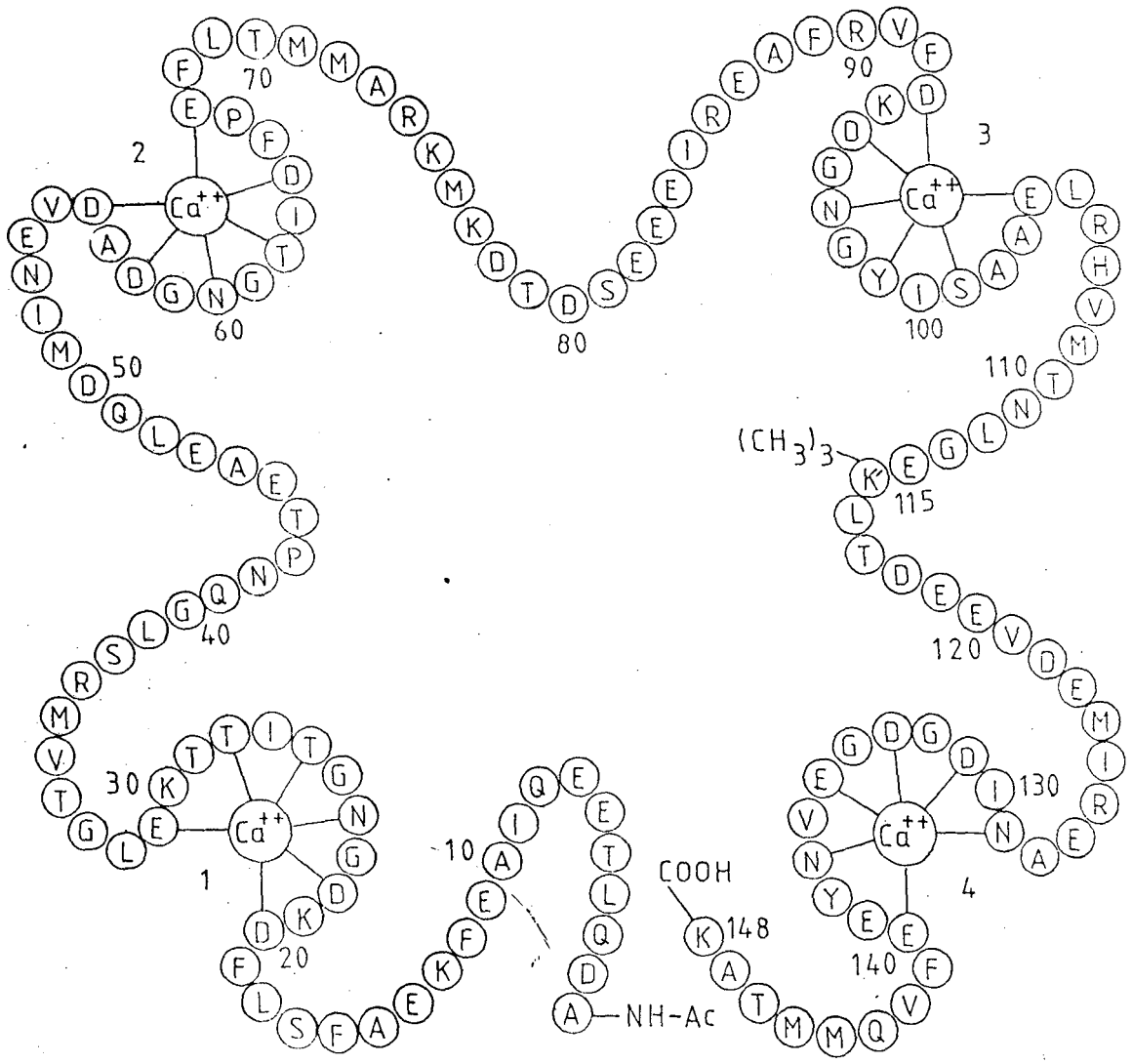

Fig.1. The complete amino acid sequence of bovine brain calmodulin as elucidated by Watterson et.al. (1980).

A = Alanine;	D = Aspartate;	E = Glutamate;
F = Phenylalanine;	G = Glycine;	H = Histidine;
I = Isoleucine;	K = Lysine;	K' = Trimethyllysine;
L = Leucine;	M = Methonine;	N = Aspargine;
P = Proline;	Q = Glutamine;	R = Arginine;
S = Threonine;	V = Valine;	Y = Tyrosine.

(From Cheung, 1982)

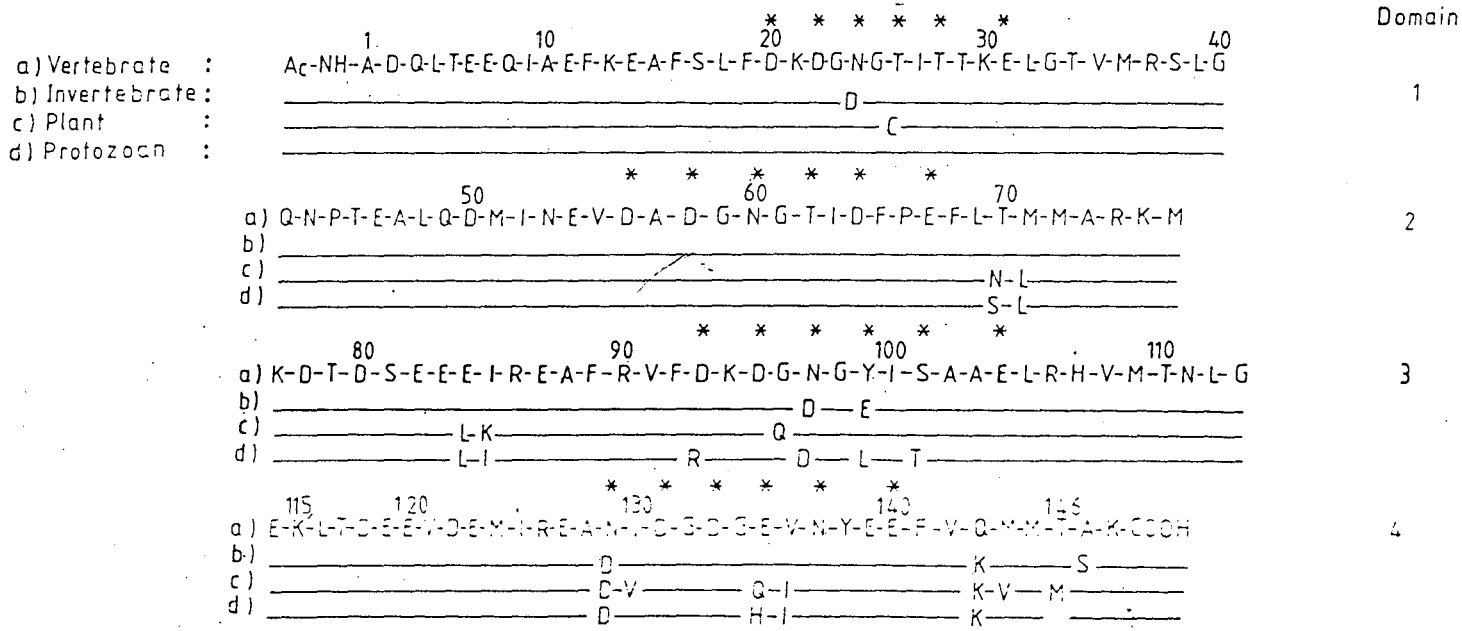

Fig.2. Comparison of amino acids sequence of Calmodulin from four phylogenitically diverse sources.
Vertebrate CaM (Bovine brain)(Watterson et.al. 1980).
Invertebrate CaM (Metridium senile) (Jamieson et.al. 1980; Takagi et.al. 1980).
Plant CaM (Spinach) (Anderson et.al. 1980; Watterson et.al.1980)
Protozoan CaM (Tetrahymena pyriformis) (Yazawa et.al. 1981).
* (Astricks) above selected amino acids indicate putative calcium liganding residues according to the model of Kretsinger (1980).
The protozoan (Tetrahymena pyriformis) CaM sequence has a deletion at position 146.
The Single Letter code for the amino acids is described in legend to Fig.1.
(From Van Eldik, 1982)

somehow endows calmodulin with unique properties to serve as an intracellular Ca^{2+} receptor. The protein is acetylated at the amino acid terminal (Fig. 1). The primary structure of all calmodulins is very similar indicating that it has been highly conserved throughout the phylogenetic scale. The internal homology in calmodulin is greatest when the first domain (residues 8-14) is aligned with the third domain (residues 81-113) and the second domain (residues 44-76) aligned with the fourth domain (residues 117-148) (Fig. 2). In as much as the four Ca^{2+} binding domains in calmodulin are nearly identical, the protein might have evolved from two doublings of a gene from an ancestral univalent Ca^{2+} binder. Each domain representing the basic E-F hand structure is flanked by two short helical regions for intracellular Ca^{2+} binding proteins (Kretsinger, 1980).

Diverse role of calmodulin in cell functions

As an intracellular receptor of Ca^{2+}, calmodulin regulates a diverse spectrum of cellular processes. Table 1 summarizes the cellular processes regulated

Table 1

Cellular processes regulated by calcium, calmodulin and Cyclic nucleotides

Cellular processes	Ca^{2+}-dependent	Role of CaM	Cyclic nucleotide
Prostaglandin synthesis	+	+	+
Smooth muscle contraction	+	+	+
Intestinal secretion	+	+	+
Insulin secretion	+	+	+
Thyroid secretion	+	+	+
Adrenal secretion	+	+	+
Neurohormone secretion	+	+	+
Dissassembly of microtubules	+	+	-
Ciliary motility	+	+	-
Fast axonal transport	+	+	-
Neurotransmitter synthesis	+	+	-
Neurotransmitter super-sensitivity	+	+	-
Cell proliferations	+	+	+
Cell architecture	+	+	+
Lysosome release	+	+	+
Histamine release	+	-	+
Lymphocyte-mediated cytotoxicity	+	+	+
Phagocytosis	+	-	+
Initiation of DNA synthesis	+	?	-

(From West, 1982).

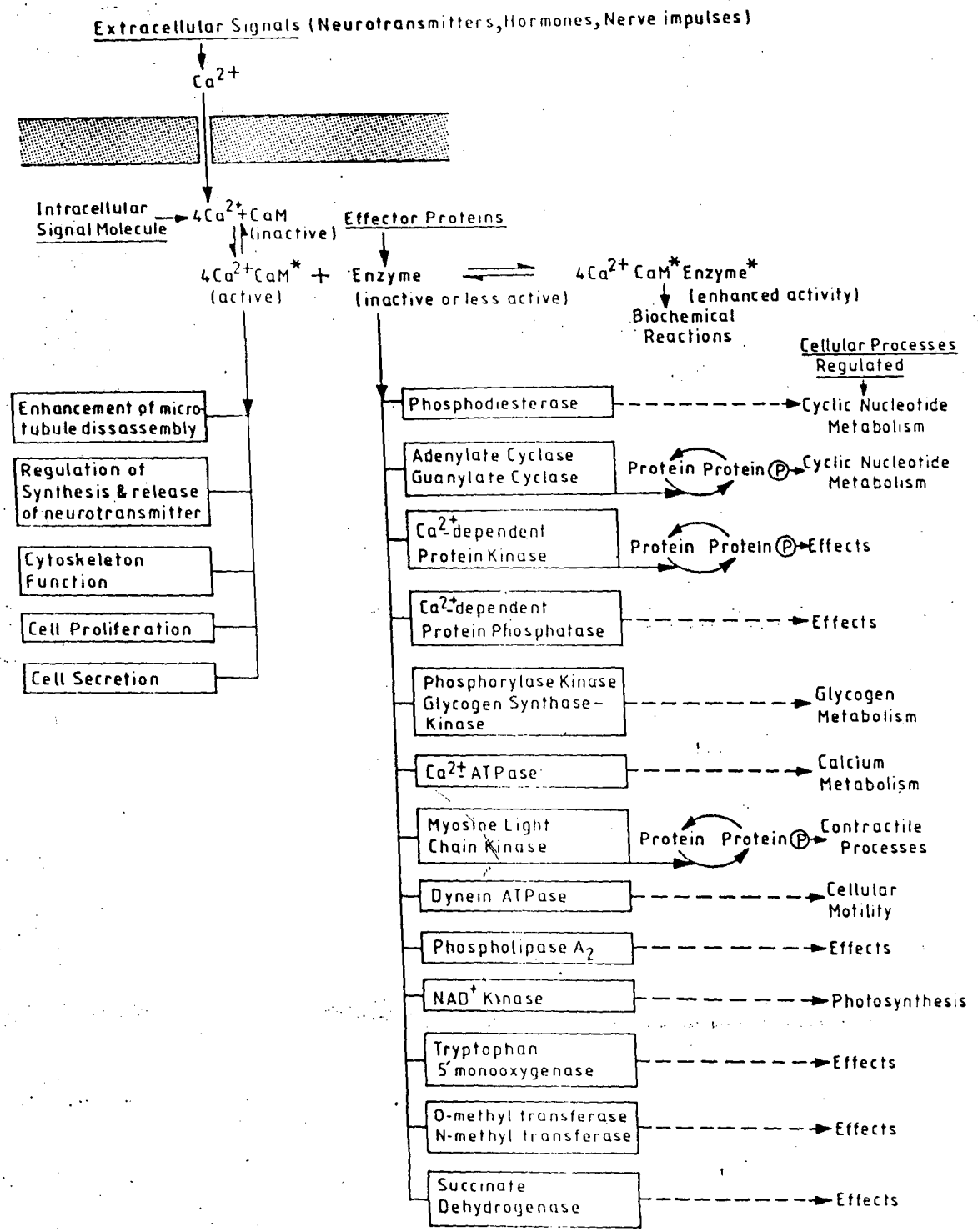

Fig.3. DIVERSE ROLE OF CALMODULIN IN CELL FUNCTIONS

(Modified from Means, 1982)

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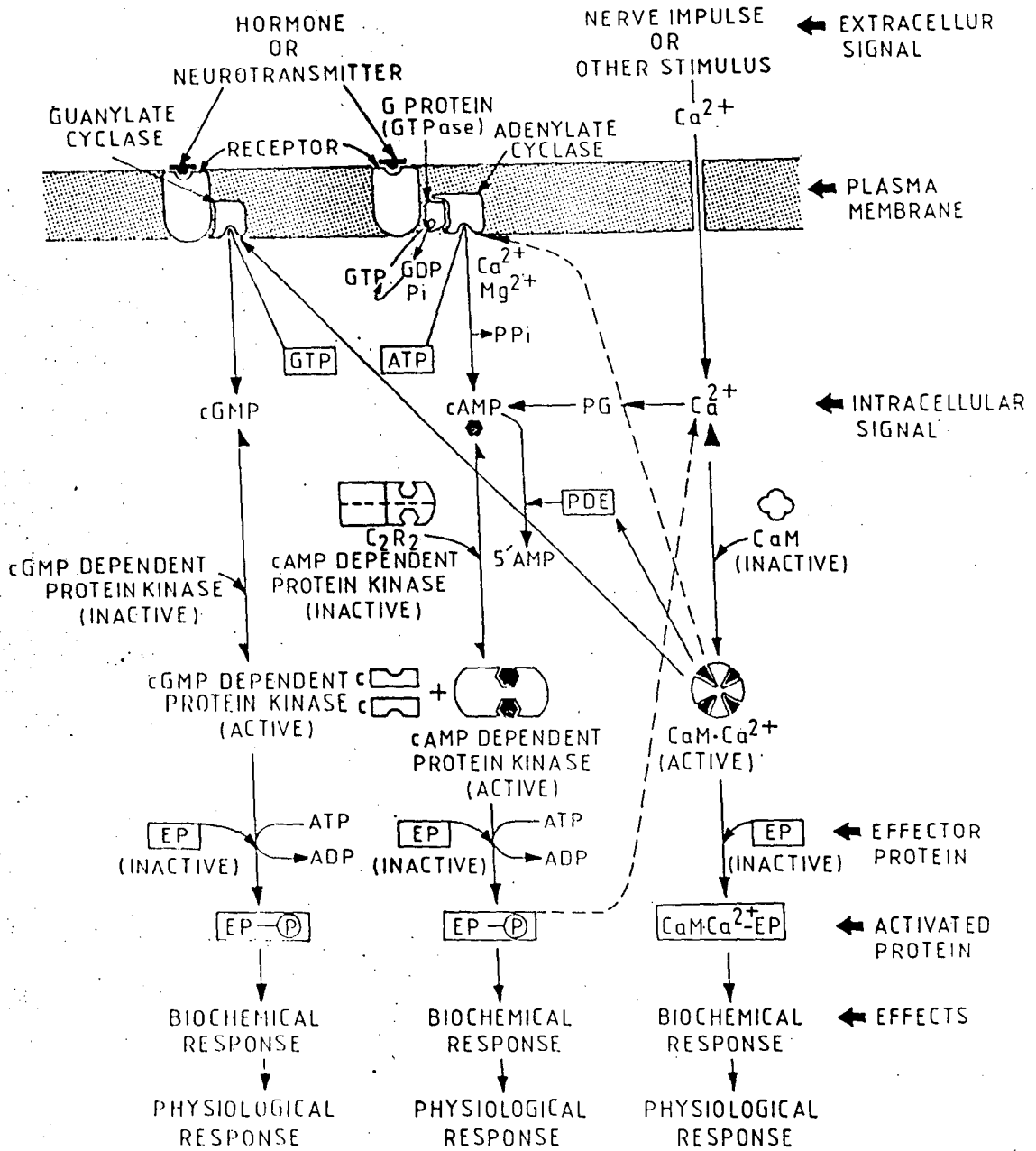

Fig. 4. INVOLVEMENT OF CALMODULIN IN CELLULAR PROCESSES AS MEDIATED THROUGH cAMP, cGMP & Ca^{2+} REGULATED PATHWAYS

cAMP = Cyclic Adenosine Mono Phosphate
cGMP = Cyclic Guanosine Mono Phosphate
CaM = Calmodulin
PDE = 3'-5' Phosphodiesterase
PG = Prostaglandin
5'AMP = 5' Adenosine Mono Phosphate
C = Catalylic sub-unit
R = Regulatory sub-unit
------ (dashed lines) indicate relationships that are still not well established.

(Modified from Nestler, 1983)

by calcium, calmodulin and cyclic nucleotaides. Fig. 3. illustrates the enzyme systems regulated and modulated by calmodulin leading to physiological responses.

Extracellular signals (first messengers) produce specific biological responses in target cells via a series of intracellular signals (second, third etc. messengers). Second messengers include cAMP, cGMP and Ca^{2+}. cAMP and cGMP produce most and possibly all of their second messenger actions through the activation of virtually one type of cAMP-dependent protein kinase and one type of cGMP-dependent protein kinase respectively. The former enzyme exhibits a broad substrate specificity and the latter a more restricted specificity. Calcium exerts many of its messenger actions through the activation of calcium-dependent protein kinases as well as through a variety of physiological effectors other than protein kinases. Calcium activates protein kinases in conjunction with calmodulin.

As illustrated in Fig. 4 cAMP, cGMP and Ca^{2+} are three major cellular messengers whose functions and metabolism are closely interwoven. Cyclic nucleotides may exert their effects in concert with or in

opposition to that of Ca^{2+}. Moreover cyclic nucleotide and calmodulin-dependent protein kinase may act on the same substrate that serves as a common effector of certain cellular processes. In controlling the metabolism and function of cAMP, cGMP and Ca^{2+}, calmodulin integrates these messenger systems on a molecular basis. Becuase the messenger systems are intertwined, cyclic nucleotides may sometimes serve as a second messenger and Ca^{2+} as the third messenger, whereas other times the roles of cAMP and Ca^{2+} may be reversed. Although the concept that both cAMP and Ca^{2+} are second messengers has been useful in understanding cellular regulation, the new insight into their interlocking and hierarchial relationships suggest that it may be more appropriate to refer them as simply messengers or regulators (Cheung, 1982).

Criteria for calmodulin regulated processes

To assess whether CaM regulates a certain biological reaction the following criteria have been suggested (Cheung, 1980):

1. The presence of sufficient calmodulin in the experimental system. This condition may be easily met because all eukaryotic cells contain adequate calmodulin.

2. The depletion of endogenous calmodulin should alter the rate of reaction and reconstitution with an exogenous calmodulin should restore the original activity.

3. The sequestration of Ca^{2+} with a chelator such as EGTA should reduce the calmodulin-induced activity to the basal value.

4. The inactivation of CaM by TFP(Trifluoperazine) or other antiphychotic drugs should reduce the CaM-dependent activity to the basal level. CaM avidly binds to these compounds in the presence of Ca^{2+} and becomes biologically inactive. In as much as these drugs are hydrophobic, however, it is necessary to ascertain that their action is due to inactivation of CaM rather than to a general hydrophobic effect.

5. Anticalmodulin should inhibit CaM-induced effect provided CaM in the test system is available to the antibody. However, the dissociation constant of an antibody to its antigen is in the range of 10^{-4} to 10^{-6}M, whereas that of CaM to its receptor protein is 10^{-9}M. In as much as the affinities of antigen-antibody complex and CaM-receptor complex are disparate, an excessive

amount of anticalmodulin may be required to exert an inhibitory effect. In practice, calcineurin,a CaM-binding protein (Cyclic nucleotide-phosphodiesterase inhibitory protein) may be a more effective reagent because it binds to CaM with an affinity of 10^{-9}M.

Characterization of calmodulin should consist of the following steps at a minimum

1. Molecular weight determination.
2. Examination of UV absorption.
3. Polyacrylamide gel electrophoresis in the presence and absence of denaturants (SDS and Urea).
4. Assay for Ca^{2+}-dependent functional properties (most commonly, activation of CaM depleted brain 3'-5'-cyclic nucleotide phosphodiesterase, PDE).
5. Amino acid analysis of acid hydrolyzed aliquots.

Inhibitors of Calmodulin

In vitro inhibition of calmodulin responses are caused by drugs: phenothiazines like TFP (trifluoperazine) (Weiss and Levin, 1978); butylphenones (Levin and Weiss, 1979) and N-(6-amino hexyl)-5-chloro naphthalesulfonamide (W-7) (Hiadaka, 1981). These agents are

known to interact with calmodulin in a Ca^{2+} dependent manner. Recent reports (Tanaka and Hidaka, 1980) have indicated that a possible binding mechanism of these agents involves a hydrophobic region in calmodulin which is exposed by a Ca^{2+} induced conformational change. Consequently these calmodulin inhibitors are used to inhibit various cell functions (Osborn, et al, 1981) in which calmodulin is involved and also to, isolate and purify calmodulin(Jamieson et al,1979). Also vinca alkoloids haloalkylamine and adrenoreceptor blockers have been shown to possess similar properties. Local anasthetics such as dibucaine, tetracaine and phenacaine and drugs such as mepacaine and propanolol with local anesthetic properties also inhibit calmodulin activities. It is not yet clear how much of the action of these drugs depend on their inhibition of calmodulin.

The present study is concerned with the investigation of calmodulin in Tetrahymena pyriformis. Tetrahymena is a well-known ciliated protozoa. It has been extensively used in diverse studies related to molecular biology, cellular biochemistry and physiology, genetics and developmental biology etc. because of ease of handling, rapid culturing, genetic stability and most important the well known method

of cell synchronization based on repeated heat shocks.

We propose to study the distribution of CaM at various cell-cycle phases of Tetrahymena. Besides, the involvement of CaM in cell division and DNA replication process, would be of great interest for a better understanding of cell-cycle progression and control mechanism operating for these important cellular events.

Purification of calmodulin in Tetrahymena

In Tetrahymena, CaM has been isolated from whole cells by four different methods:

1. Based on Ca^{2+} - dependent affinity chromatography on Phenothiazine (CAPP 10 chloro- (3-aminopropyl) phenothiazine)-Sepharose 4B (Jamieson, et al 1979).
2. Based on the use of Tubulin-Sepharose affinity column (Kumagai, et al, 1980).
3. Based on trichloroacetic acid (TCA) precipitation and SDS-polyacrylamide gel electrophoresis (Kakiuchi et al., 1981).
4. Based on electrophoretic characteristics as guide (Suzuki et al, 1981).

Table 2

Sub-cellular distribution of calmodulin in Tetrahymena*

Sub-cellular fraction	Calmodulin	
	µg CaM/mg protein	% of total
Cilia	1.3	1.0
Ciliary supernatant	6.7	43.0
Pellicles	0.1	2.7
Mitochondria	0.2	2.2
Microsomes	0.07	1.3
Post microsomal fraction	1.0	50
	0.86 = Total amount of CaM / Total amount of protein	100.2

* Nagao, et al, 1981.

Table 3

Effects of Calmodulin from Tetrahymena and Bovine brain on various calmodulin -dependent enzymes

Enzyme	CaM minus	Tetrahymena CaM	Bovine brain CaM
Guanylate cyclase (Tetrahymena)	40	1330	41
	($p.$ mol.min^{-1}, mg $protein^{-1}$)		
Phosphodiesterase	72.6	190.5	203.5
	($p.mol.min^{-1}$. mg $protein^{-1}$)		
Adenylate cyclase (rat brain)	273.7	385.7	448.5
	($p.mol.min^{-1}$.mg. $protein^{-1}$)		
Myosine light-chain kinase (chicken gizzard)	1.0	7.3	8.1
		($p.mol.min^{-1}$)	
$(Ca^{2+} + Mg^{2+})$-ATPase	1.78	3.40	3.56
		(Pi.μ. mole/60 min)	
NAD^{+}kinase (Pisum sativum)	3.0	37.2	40.9
		(u /min)	

u = One arbitary unit defined as decrease in 0.01 absorbancy at 570 nm. Enzyme activities assayed in the presence or absence of CaM (5 µg/assay tube) with 50 µM Ca^{2+} (Kudo et al., 1982).

1983). Postulating that the ability of mammalian cell CaM to activate guanylate cyclase would be lost during the course of evolution, it can be considered that Tetrahymena CaM could be an ancestral form of mammalian CaM. However, in general, calmodulin-dependent enzymes such as PDEase and myosine light chain kinase can be activated by CaM from any source, but the activation of guanylate cyclase by CaM is limited to the protozoan calmodulins (Kudo, et al, 1981). Guanylate cyclase in Tetrahymena per se seems to be specific. In Tetrahymena cells there are at least two types of endogenous calmodulin sensitive enzymes. One is guanylate cyclase and the other is dyenin ATPase in the ciliary fraction of the cell.

Tetrahymena and bovine brain CaM are activated by CaM-deficient brain phosphodiesterase in the presenee of Ca^{2+}. Although the extent of the maximal activation of phosphodiesterase produced by Tetrahymena CaM is comparable to that of bovine brain CaM, the amount of CaM required for half maximal activation of the enzyme differed (Nagao et al., 1981).

In the activation by phosphodiesterase, the potency of Tetrahymena CaM represented by the reciprocals of the quantity of CaM required for half-maximal

Thus there are two major pools of calmodulin in Tetrahymena cells i.e. ciliary supernatant and post microsomal fractions. This is now evident that these two pools are metabolically isolated from each other to some extent (Nagao, et al, 1981).

The amino acid sequence of Tetrahymena pyriformis CaM has a deletion at position 146. There are only 13 differences between Tetrahymena and bovine brain calmodulin. Like the invertebrate calmodulins the majority of the sequence differences are found in domains 3 and 4 (Fig. 2). These few differences are interesting since Tetrahymena guanylate cyclase is activated by Tetrahymena CaM but not by vertebrate or other invertebrate CaM (Kakuichi et al, 1981). However, Tetrahymena CaM will activate vertebrate phosphodiesterase (Jamieson et al, 1979; Kakuichi et al., 1981) indicating that there may be species specificity in some colmodulin enzyme interactions.

As shown in Table 3 Tetrahymena CaM is almost as effective as bovine brain CaM in activating many CaM dependent enzymes. A particularly specific feature of Tetrahymena CaM is to be able to activate guanylate cyclase, but not adenylate cyclase (Nagao,

activation of enzyme is 22.7% of bovine brain CaM. The concentration of Ca^{2+} required for half-maximal activation of enzyme is 2 and 4 μM with bovine brain and Tetrahymena respectively (Inagaki, et al, 1983).

Thus, Tetrahymena CaM seems to be of great interest and our investigation is aimed at eliciting some more information regarding its involvement during cell cycle traverse.

MATERIALS AND METHODS

Cell and culture technique

Tetrahymena pyriformis (W) (amicronucleate strain) was used as the experimental system. The cells were grown in 2% Proteose-peptone medium enriched with 0.02% liver extract (PPL medium) axenically at 28°C. PPL medium was prepared by dissolving 20 gms of Proteose-peptone and 0.2 gm of Hog liver extract in one litre of double distilled water by heating the medium. After filtering the medium through a sintered glass filter (G_2), 100 ml of the medium was poured into 250 ml flasks and autoclaved at 15 lbs psi for 20 minutes. Flasks were innoculated aseptically with 0.1 ml of broth containing ca.1-2 x 10^5 cells/ml with the aid of a sterile transfer pipette and allowed to grow at 28°C.

Cell synchronization

After a period of logarithmic growth lasting for atleast 15 hrs at 28°C, the cells were synchronized using 'multi-heat shock method' of Scherbaum and Zeuthen (1954). The six temperature cycles that followed consisted of 30 minutes at 34°C and 30 minutes at 28°C. At the end of the heat shock (EHS), a sample was taken for determining the cell density with the

help of a haemocytometer. Incubation was allowed to continue at 28°C for the duration of the experiment with sampling to determine the onset and duration of cell-division and mitotic-indices. Synchronous cell division was observed at about EHS + 80 minutes and again at EHS +260 minutes after the final heat shock. A third cycle of division although less well defined than the first two was also observed.

Determination of mitotic index

After synchronization, at time. EHS + 80 minites, when the cells are in mitotic phase, sample of cells were removed and fixed in acetic acid : ethanol (1:3 v/v) using equal volume of cell suspension and fixative. 1 ml of fixed cells were introduced into the counting chamber of haemocytometer. Under the microscope, the number of dividing cells and the total number of cells were counted per unit area of the grid (1 sq mm). The mitotic index (M.I.) was calculated by following the equation:

$$\text{Mitotic index (M.I.)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

Each experiment was repeated atleast three times and the mean values were obtained from 10 observations from each slide.

Cell cycle kinetics of Tetrahymena pyriformis

The duration of cell-cycle phases (i.e. M, G_1, S and G_2) was determined by pulse labelling technique with ^{3}H-thymidine (^{3}H-TdR). Cell suspension, synchronized as described before, was incubated at 28°C. At EHS + 80 minutes (t = 0 for the experiment), the cells were observed for dividers taking a sample from the suspension of cells and another sample was taken for determining the cell-density. 0.2 ml of samples were removed at 20 minutes interval in triplicate and 50 ul of ^{3}H-TdR 100 uCi/ml (sp. activity 17.8 mCi/mm) were added. After 10 minutes, each sample was chased with washing medium (Hamburger and Zeuthen, 1957) having a composition of : KH_2PO_4-15gm, K_2HPO_4-0.68gm, NaCl-2.75gm; $MgSO_4.7H_2O$-0.25gm, double distilled water to make one litre, and excess of unlabelled thymidine (2mM) at 4°C. Then the samples were washed on presoaked millipore filter papers (GF/C, pore size 0.45 um) with unlabelled precursor and then blotted dry, using a filtration assembly. This washing was repeated twice. Then the cells were disrupted with 15% TCA followed by 95% ethanol at 4°C. The GF/C millipore filters having cold TCA insoluble precipate after drying were transferred into scintillation vials and

further dried in an oven at 60°C for one hour. 8 ml of toluene based scintillation fluid having the following composition (PPO-2gm, POPOP-0.5gm and Toluene-500 ml) was added in each scintillation vial. Samples were assayed for radioactivity in Beckman Liquid Scintillation counter (efficiency of counter= 63%) for 2 minutes for 2 cycles in each vial with quench correction to get CMP values.

Quantitation of calmodulin levels during different phases of cell cycle

Cell homogenization

Cell suspensions of Tetrahymena were synchronized by repeated heat shock method of Scherbaum and Zeuthen (1954) and cells were harvested by pelleting in conical centrifuge tubes at 1400 xg. 10 ml packed volume of cells were collected from the mid-M, mid-G_1, mid-S and mid-G_2 phases of the cell cycle. Cells were washed once with 0.9% NaCl at 4°C and centrifuged at 1400 xg for 3 minutes. The supernatant was discarded and the pellet was stored at -20°C. After thawing, the cells were homogenized in ultrasonic disintegrator (MSE) in 2 volumes of homogenization buffer (20 mM TES NaOH/ 1 mM EDTA/1 mM-2-meracaptoethanol/ 1 mM PMSF, pH 7.0) for 30 seconds at 4°C. All subsequent procedures were carried out at 4°C unless specified otherwise.

Ammonium sulfate precipitation

The homogenate was heat treated at 85°C for 5 minutes, by keeping the beaker in a water bath maintained at 85°C and immediately cooling to 0°C by keeping the beaker in ice. The homogenate was then centrifuged at 11000 xg for 60 minutes and the supernatant decanted into a beaker and solid ammonium sulfate (351 gm/litre) was added to bring the solution to 55% saturation. The resulting solution was adjusted to pH 7.0 with 1 M NH_4OH and stirred for one hour. It was then centrifuged at 12,000 xg for 30 minutes. To the supernatant 1M Mg $(CH_3COO)_2$ was added to make the final concentration 10 mM and the supernatant was brought to pH 4.0 by slow addition of 0.5 M H_2SO_4 and the mixture was stirred for 2 hours. The material precipitated by this procedure was collected by centrifugation at 12000 xg for 30 minutes at 4°C. The supernatant was discarded. The pellet was resuspended in 2 ml of homogenization buffer and 0.1 M $CaCl_2$ was dialysed against 0.01 M ammonium bicarbonate, frozen and lypholized.

Electrophoresis (SDS-PAGE)

Calmodulin's small size (M_R = 16.7 Kd) and low PI($\simeq$ 4) makes it ideally suited for electrophoretic

analysis at neutral to alkaline pH (Vanaman, 1983). Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Watterson (1976) using tube gels (0.4 x 12 cms).

Gel Preparations

(a) Preparation of separating gels: The separating gels of 10% acrylamide were prepared by mixing solutions (A), (B), (C) and (D) in the ratio 1:2:4:1. Solution (A) contained 36.3 gm Tris, 46 ml of IN HCl and 0.26 ml TEMED and distilled water to make the volume upto 100 ml. Solution (B) contained 40 gm acrylamide and 1.2 gm bis-acrylamide and distilled water to make the volume 100 ml. Solution(C) contained 0.14 gm of ammonium per sulfate in 100 ml of distilled water. Solution (D) contained 0.8 gm SDS in 100 ml distilled water.

(b) Preparation of stacking gel: The stacking gels of 4% acrylamide were prepared by mixing solutions (i), (ii), (iii) and (iv) and distilled water in the ratio of 1:1:4:1:1. The solution (i) contained 5.98 gm Tris, 48 ml of IN HCl and distilled water to make 100 ml. Solution (ii) contained 10 gm of acrylamide and 2.5 gm

of bis-acrylamide and distilled water to make upto 100 ml. Solution (iii) contained 40 gm of sucrose in 100 ml of distilled water. Solution (iv) contained 40 mg riboflavin in 100 ml of distilled water.

The solution mixture of (A), (B),(C) and (D) was stirred and poured in the get tubes. A drop of water was layered on the top of the gels and the gels were then allowed to polymerize for one hour. After polymerization, the water was removed with tissue paper and the stacking gel mixture prepared by mixing (i), (ii), (iii) and (iv) and distilled water was poured over the separating gel. A drop of water was layered on the top of the gel and allowed to polymerize for 4 hours.

(c) Sample loading and electrophoresis: The upper reservoir (cathode compartment) contained 0.05M Tris base 1mM EDTA, 0.4%(w/v) glycine, 0.07%(v/v) 2-mercaptoethanol (pH 8.4). The lower reservoir (anode compartment) contained 0.1 M Tris base. o.05M HCl (pH 6.6). Samples were dissolved in 2ml of upper reservoir buffer. Aliquot of sample was mixed with an equal volume of 0.01%(w/v) bromophenol blue in 50% glycerol(w/v). To this 1% SDS was added to

make the final concentration 0.1% of SDS. Electrophoresis was performed at 150 volts with a constant current of 3mA per gel for 5 to 7 hours.

(d) Fixation, staining and destaining of gels: Gels were fixed in fixative (prepared by mixing 150ml methanol and 350 ml distilled water and then adding 17.25 gm sulphosalicylic acid and 57.5gm TCA) for 30 minutes. Subsequently, the gels were stained with 0.25%(w/v) Coomassie brilliant blue R250 in 50%(v/v) methanol and 10% acetic acid glacial for 30 minutes at 37°C and destained in 7.5 (v/v) acetic acid glacial and 5%(v/v) methanol at 37°C for more than 24 hours.

Gel scanning was done in a Joyce Lobel Chromoscan 200 at 620 nm with 1:1 speed. CaM was quantitated in homogenates by comparison of integrated areas of calmodulin protein peaks in gel scans with those of bovine brain calmodulin (M.wt.16.7 kd) as standard.

Protein estimation

Total protein was estimated by Bradford's method (Bradford, 1976). 0.1 ml of the homogenized solutions from each phase of cell cycle of Tetrahymena were diluted to known sample volumes. 0.1 ml of the diluted homogenates were then transferred to test-tubes

in triplicates and 5ml of Bradford solution (prepared by dissolving 100 mg Coomassie Blue G-250 in 50 ml of 95% ethanol, 100 ml of 85%(v/v) orthophosphoric acid and the solution was made upto one litre with distilled water) was added in each test tube. Absorbance at 595 nm was recorded and the amount of total protein was calculated comparing the standard curve obtained using bovine serum albumin (BSA) as standard.

Calmodulin and Calcium inhibition

a) Effect of TFP on M phase: At (EHS + 70 min) i.e. 10 minutes prior to the onset of M phase at the end of the last heat shock, when the cells were being incubated at 28°C, trifluoperazine (TFP) was added to bring the final concentration of 18 μm of TFP. After 10 minutes treatment, the cells were fixed in acetic acid glacial: ethanol (1:3) mixture at EHS + 80 min., untreated cells were kept as controls. Both treated as well as untreated cells were placed on the subbed slides. The slides were dehydrated in 100% and 90% ethanol and stained with giemsa stain buffered with phosphate buffer (pH 7.0). Under the mocroscope the number of dividing cells and total number of cells were counted per unit area of the eye piece graticule in the ocular and the mitotic indices were calculated in both cases.

b) Effect of EGTA and TFP on G_1/S phase: To evaluate the effect of EGTA which is a specific calcium chelator and TFP which is an inhibitor of calmodulin on the initiation of DNA synthesis the following experiments were performed. At EHS + 155 minutes which is the time 10 minutes prior to G_1/S boundary after synchronization, 4.5 ml of samples were transferred in 4 sets of test tubes in duplicate. To the first set 0.5 ml of distilled water was added, which acted as control. To the second set 0.5 ml of 20 mM EGTA was added to bring the final concentration to 2mM EGTA. To the third set 0.5 ml of 180 μM TFP was added to bring the final concentration upto 18 μM TFP. To the fourth set EGTA and TFP were added to final concentration of 2 mM EGTA and 18 μM TFP. After 10 minutes of treatment 0.2 ml of treated cell suspensions were removed in test tubes and 50 μl of 100 μCi ^{3}H-TdR was added in each test tube. After 10 minutes of pulse labelling, each sample was chased with washing medium containing excess of unlabelled thymidine (2mM) at 4°C. The samples were transferred on to the millipore filter GF/C presoaked and blotted dry and washed the samples twice with washing medium containing unlabelled TdR. Subsequently the cells were disrupted

with 15% TCA at 4°C and washed thrice with 5% TCA, followed by 95% ethanol at 4°C. The GF/C millipore filters having TCA insoluble residue after drying were transferred into scintillation vials and further dried in over at 60°C for one hour. 8 ml of toluene based scintillation fluid was added in each vial. Samples were then assayed for radioactivity.

c) Effect of EGTA and TFP on the uptake of ^{3}H-TdR

To evaluate the effect of EGTA and TFP on the uptake of ^{3}H-TdR at EHS + 155 min. 1.8 ml samples were transferred to 4 sets of test tubes in duplicate. To the first set 0.2 ml of distilled water was added which acted as control. To the second set 0.2 ml of 20 mM EGTA was added. To the third set 0.2 ml of 180 µM TFP was added. To the fourth set TFP and EGTA were added to final concentration of 2 mM EGTA and 18 µM TFP. After 10 minutes of treatment 200 µl of 100 µCi ^{3}H-TdR was added in each test tube. After 10 minutes of pulse labelling, 5 ml of washing medium containing excess of unlabelled thymidine was added in each test tube and centrifuged. The supernatant was discarded and the pellet was washed twice with same washing buffer. The cells were lysed by adding 0.5 ml of 1% triton-X into each tube and transferred into scintillation vial

containing 8 ml of toluene based scintillation fluid and assayed for radioactivity.

Cytological preparations and photomicrography:

To ascertain the efficiency of synchronization, the permanent slides were prepared as follows. At EHS + 80 min after six repeated heat shocks as mentioned before, cells were immediately fixed in acetic acid: ethanol (1:3 v/v) for 20 minutes on subbed slides (prepared with 0.5% gelatin and 0.05% chrome alum). The slides were dehydrated in 100% and 90% ethanol for 10 minutes each, stained with phosphate buffered giemsa stain (pH 7.0) and mounted with DPX. All light microscopic observations were made and photomicrographs were taken with the help of a Carl Zeiss photomicroscope-II.

Source of Chemicals:

Proteose-peptone (Difco certified) was obtained from Difco Laboratories, Detroit, Michigan, USA. Hog Liver extract (crystallized powder), BSA, TES, Tris, PMSF, PPO, POPOP, Thymidine (unlabelled), Triton X, TFP, EGTA, Sucrose, Riboflavin (Vitamin B_{12}), Coomassie Brilliant Blue G-250, sulfosalicylic acid and magnesium acetate were obtained from Sigma Chemical Company, USA. Acrylamide (recrystallized from chloroform), bis-acrylamide, TEMED, SDS, ammonium per sulfate, Coomassie brilliant blue R-250 were obtained from Bio-Rad Laboratories, USA. Giemsa stain was procured from British Drug House, Poole, England. ^{3}H-TdR (Thymidine (methyl-T) (Specific activity 17.8 mCi/mm) was obtained from the Isotope division of Bhabha Atomic Research Centre, Bombay, India. Gelatin (bacteriological), TCA, Toluene, $MgSO_4.7H_2O$, NaCl, EDTA (disodium), H_2SO_4, HCl, DPX, were obtained from BDH and were of analytical grade. Ethanol was obtained from Bengal Chemicals, Calcutta. KH_2PO_4, K_2HPO_4(anhydrous), $CaCl_2.2H_2O$, $(NH_4)_2SO_4$ and glycerol were obtained from Sarabhai Chemicals, India and were of analytical grade. 2-mercaptoethanol and Bromophenol Blue were obtained from E.Merck, Germany.

EXPERIMENTAL RESULTS AND OBSERVATIONS

Heat-shock induced cell-synchrony

To determine the levels of CaM throughout the cell-cycle of Tetrahymena pyriformis it is necessary to find out first the mean generation time and the duration of the cell-cycle phases. Multiple heat-shock procedure comprising of growing the cells at 28°C and alternating with elevating the temperature to 34°C at half an hour intervals results in mitotic cell synchrony in Tetrahymena. When 18 hours old Tetrahymena pyroformis cultures having a cell density of ca. 2 x 10^5 cells/ml were thus subjected to a series of six heat shocks, there was an initiation of synchronous cell-division at around 85 minutes after the end of the last heat shock. When dividing populations from different synchronous cultures were scored for the mitotic index, a value of ca 90% was obtained and thus the starting synchronized cell population was comprised of 90% mitotic cells at time zero (Fig. 6). After the completion of the first cell cycle, a second synchronous mitotic division occured after an interval of 184 minutes. This period constituted the cell duplication time of synchronous Tetrahymena population. It was also observed that the index of mitotic synchrony was appreciably lower in the second synchronous divisions.

Fig. 5. a) Photomicrograph of Tetrahymena pyriformis from asynchronous culture grown at 28°C. Cells fixed and stained with giemsa x 112.

b) Magnified view of the same x 220.

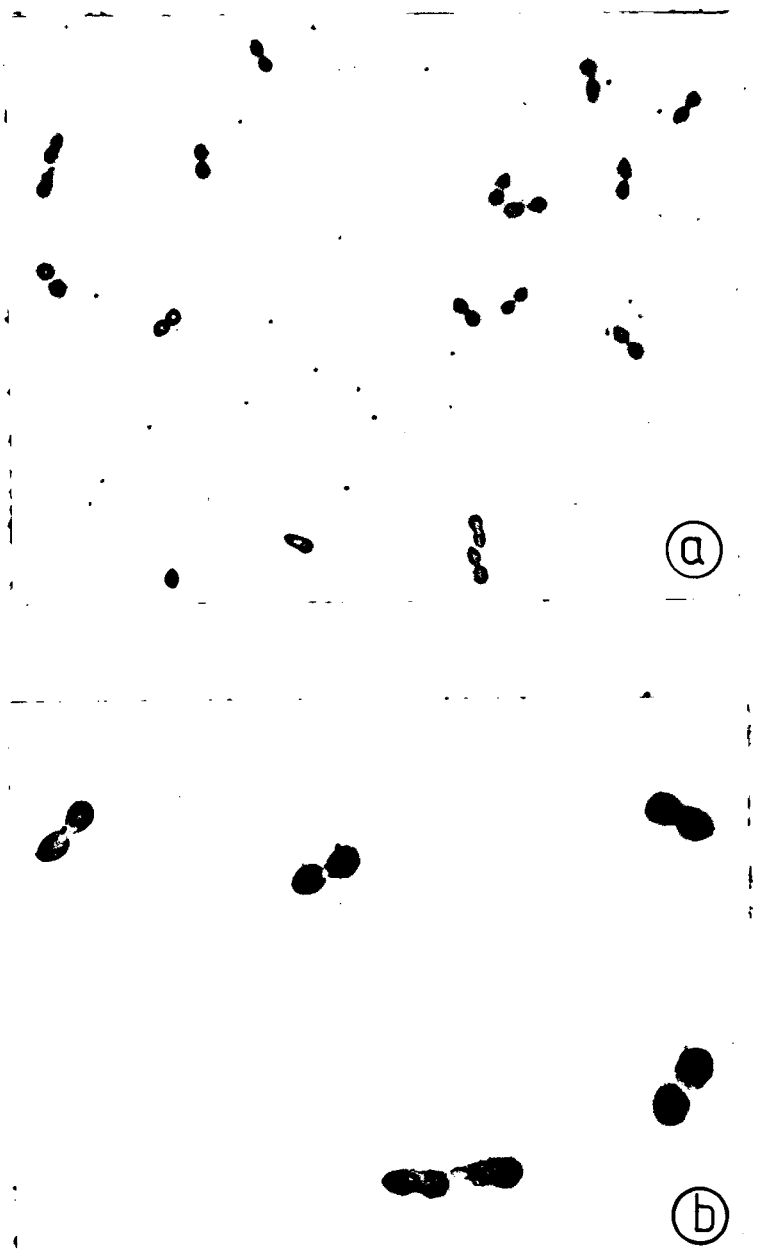

Fig. 6. a) Photomicrograph of Tetrahymena pyriformis from heat-shock induced synchronous cultures. Note the synchronously dividing ciliates x 69.

b) Magnified view of the same x 394.

Duration of cell-cycle phases

The duration of different cell-cycle phases of the synchronized Tetrahymena cell populations was determined by pulse labelling with ^{3}H-thymidine (^{3}H-TdR) for 10 minutes at periodic intervals. The duration of M phase was ascertained by the time taken by mitotic cells to form two daughter cells and this was found to be about 20 minutes. With the progression of the daughter cells in the next phase, there is a gradual increase in ^{3}H-TdR incorporation after a gap of about 60 minutes (G_1 phase) and maximum incorporation of ^{3}H-TdR occurred during a period of 40 minutes (S phase). A steep decline in ^{3}H-TdR incorporation occurred thereafter and continued for about 60 minutes (G_2 phase) before the onset of next synchronous division. The cell-cycle timings of Tetrahymena pyriformis population have been depicted in Fig. 7.

CaM levels during different phases of cell-cycle

The relative levels of CaM at various cell-cycle phases in the synchronized cell populations have been shown in Table 4. The average CaM concentration in the mid-phase of M was 0.742 µg of CaM/mg protein. For the mid-G_1 phase, the concentration

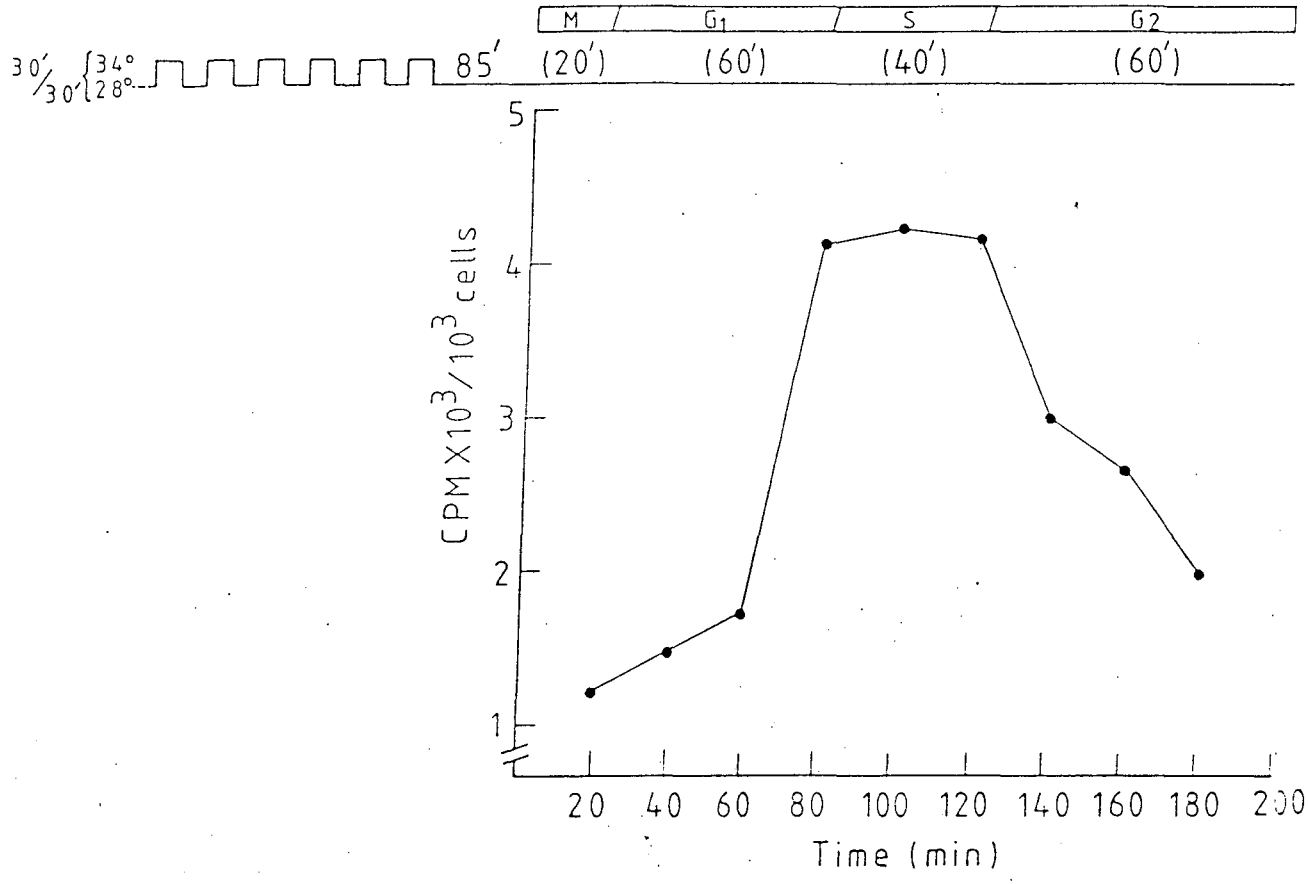

Fig. 7. Cell cycle phases of Tetrahymena pyriformis (W). The incorporation of acid insoluble fraction of ^{3}H-TdR into nuclear DNA has been assayed by pulse labelling the cells at periodic intervals.

Table 4

	µg of CaM/mg of protein			
	M	G_1	S	G_2
Experiment 1	0.88	0.34	0.88	0.83
Experiment 2	0.70	0.32	0.74	0.76
Experiment 3	0.64	0.30	0.75	0.74
$\bar{X} \pm$ S.D	0.74±0.124	0.32±0.015	0.79±0.078	0.79±0.047

Discontinuous SDS-PAGE was performed for the quantitation of CaM in supernatant fraction from partially purified homogenates of Tetrahymena from different phases of the cell-cycle. Equal aliquots from each homogenate supernatant fraction were loaded on the acrylamide gels. The CaM present in homogenate supernatants from different phases of cell cycle was quantitated by comparison of the integrated areas of CaM peaks in the scans of gels containing known amounts of purified Bovine brain CaM to those obtained for known aliquots of supernatants of Tetrahymena homogenates from different phases of the cell-cycle.

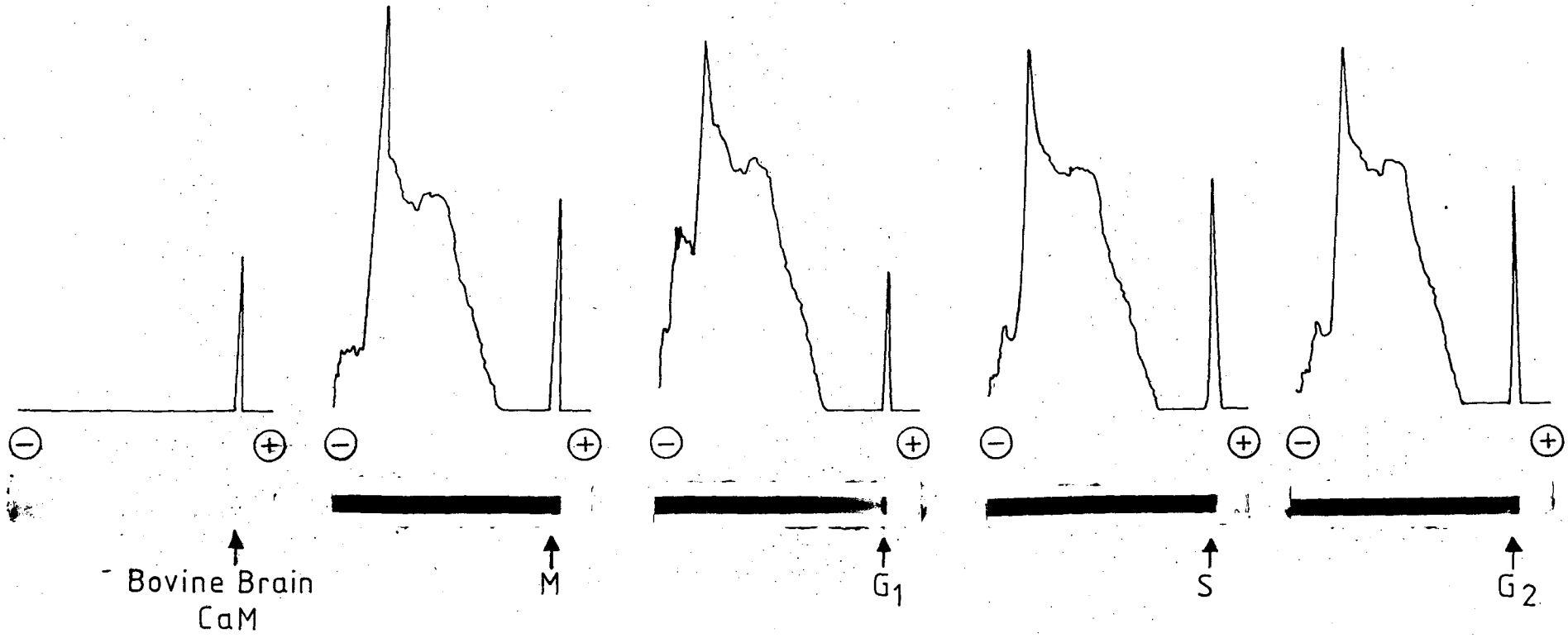

Fig. 8. SDS-PAGE photograph of the gels and its chnomoscan showing the profile of Calmodulin from different phases of cell-cycle of Tetrahymena pyriformis (from Experiment No.1).

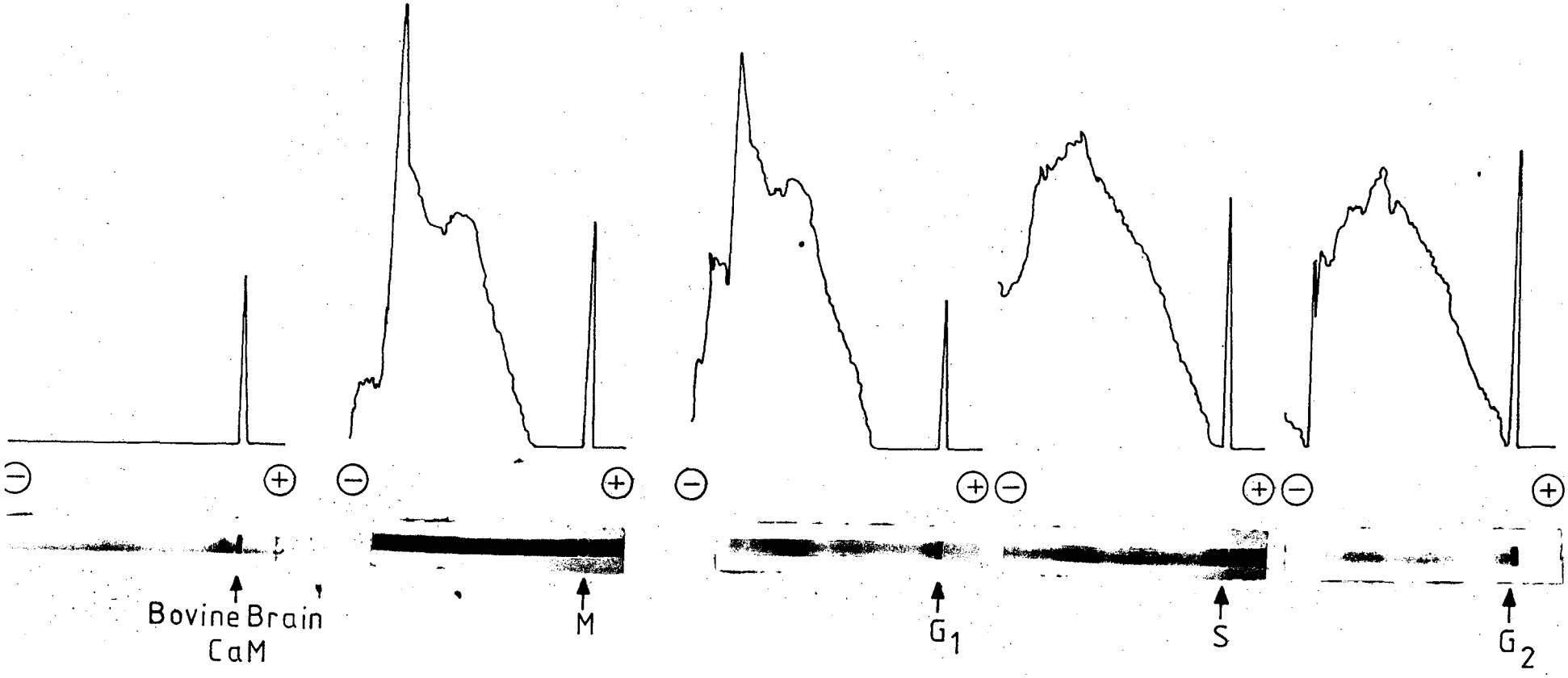

Fig. 9. SDS-PAGE photograph of the gels and its chromoscan showing the profile of Calmodulin from different phases of cell-cycle of Tetrahymena pyriformis (from Experiment No.2).

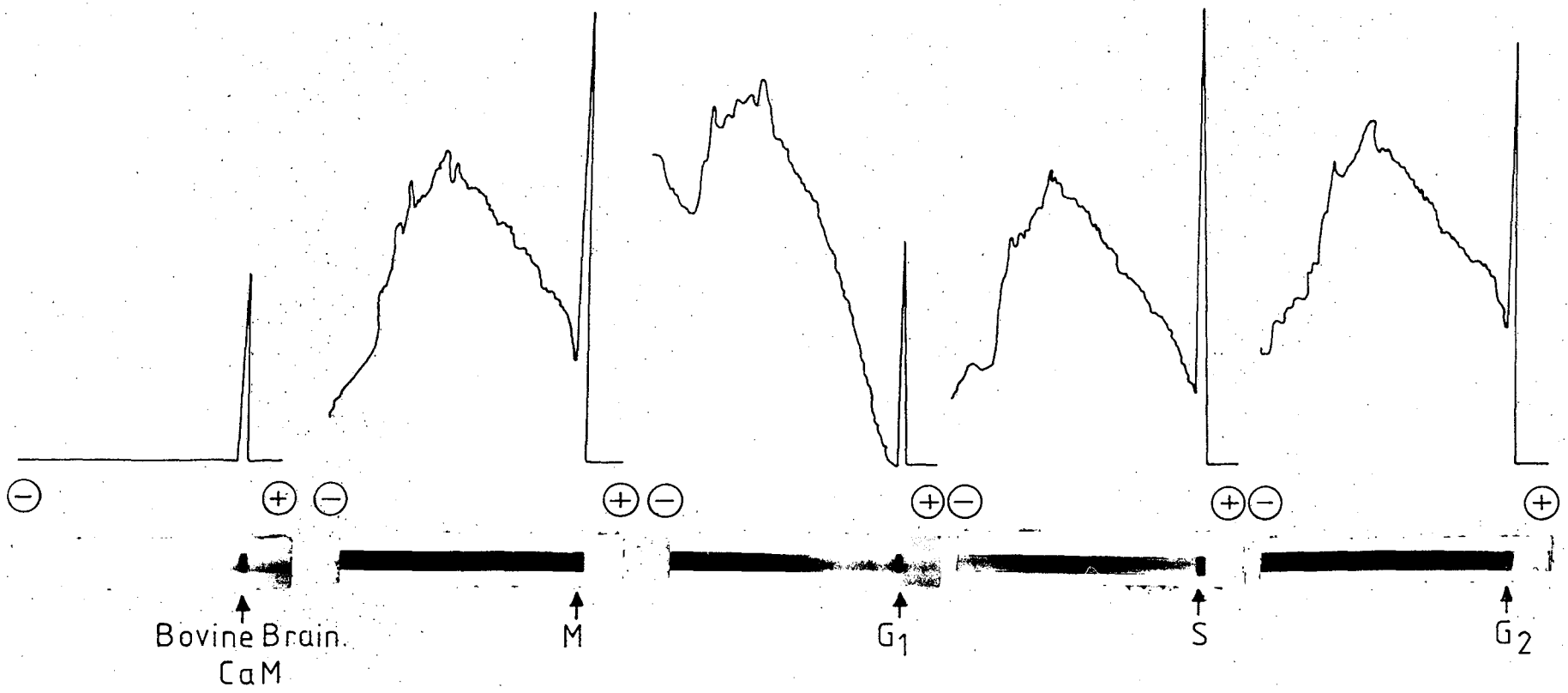

Fig. 10. SDS-PAGE photograph of the gels and its chromoscan showing the profile of Calmodulin from different phases of cell-cycle of Tetrahymena pyriformis (from Experiment No.3).

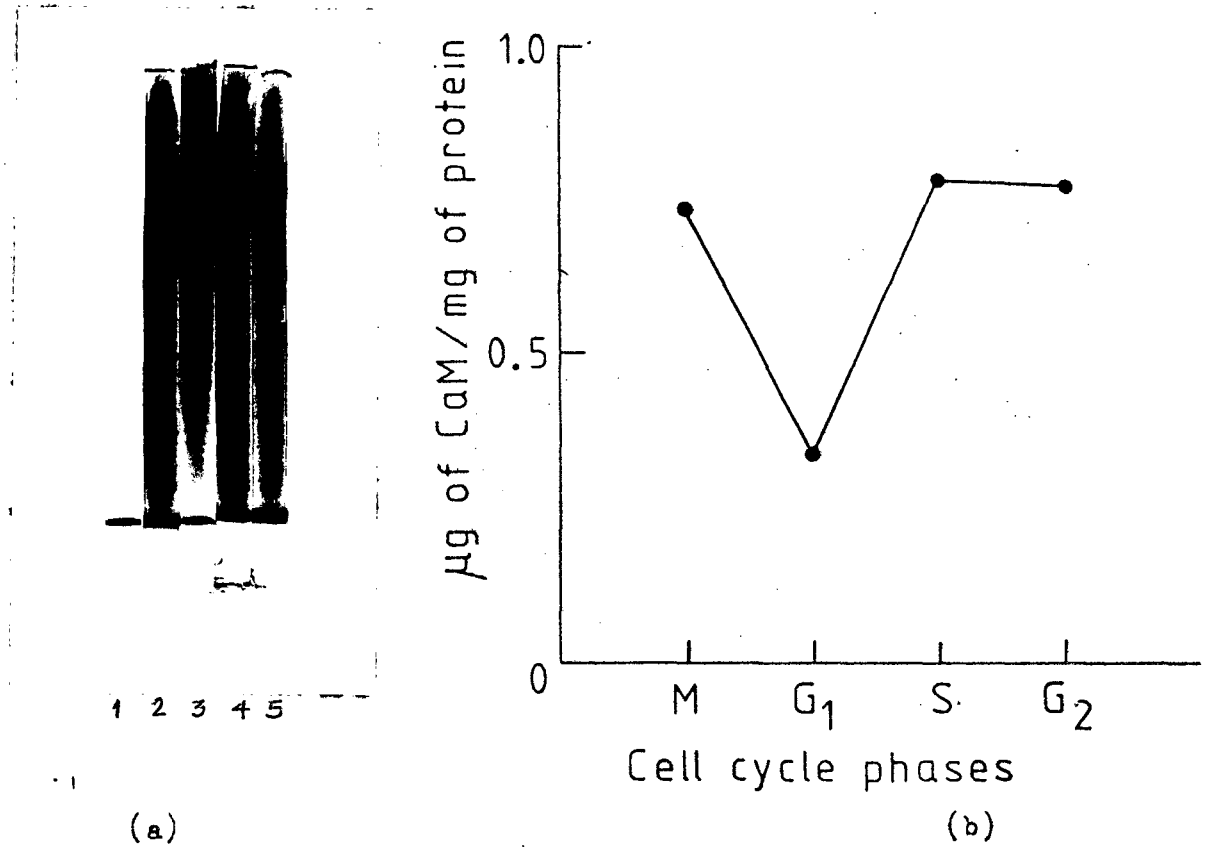

Fig. 11. The levels of Calmodulin at different phases of cell-cycle in Tetrahymena pyriformis(W). The Calmodulin concentration has been quantitated at mid of different phases of cell-cycle.

a. SDS-PAGE Profile of Calmodulin

b. Densitometric quantitation of the same.

1 = Bovine brain CaM ; 2 = M ; 3 = G_1 , 4 = S and 5 = G_2 .

of CaM was found to be 0.323 µg of CaM/mg protein, showing that the concentration of CaM was reduced to less than half that of obtained in M phase. During mid-S, the concentration of CaM was 0.790 µg of CaM/mg protein which was comparable to the level at mid-M. In the mid-G_2, the concentration of CaM was 0.776 µg of CaM/mg protein, showing that the level of CaM at G_2 was equal to the level of S, and M. So our experimental results showed that in Tetrahymena at G_1 phase of cell cycle, the CaM is reduced to less than 50% level as compared to the other phases of the cell cycle (Fig. 8,9 and 10). The summary of different experiments on the levels of CaM at various cell-cycle phase has been shown in Fig. 11.

Effect of inhibitors of Ca^{2+} and CaM on DNA synthesis and mitosis

Since CaM has been shown to be involved in various important cellular functions, we wanted to assess the effect of inhibitors of CaM as well as of Ca^{2+}. Trifluoperazine (TFP) is a phenothiazine derivative which is used as an antipsychotic drug, has been shown to inhibit selectively CaM activation of phosphodiesterase in a calcium dependent fashion (Levin and Weiss, 1977), Satir et al (1980) reported

that 18 μM TFP was capable of inhibiting the intracellular CaM of Tetrahymena. 2mM EGTA, which is a well known chelator of Ca^{2+} was used to inhibit Ca^{2+} in Tetrahymena cells.

a) In order to test the hypothesis that CaM is involved in the initiation of DNA synthesis, the cells at G_1/S boundary (EHS +165 minutes) were treated with TFP for 10 minutes and it was found that the incorporation of ^{3}H-TdR was reduced to about 45% as compared to the untreated cells. Further the treatment with EGTA at this point of cell-cycle showed the decrease in the incorporation of ^{3}H-TdR but the reduction was about 20%. When TFP and EGTA both were applied simultaneously there was 72% decrease in the incorporation of ^{3}H-TdR during the S phase of Tetrahymena (Fig. 12a).

b) In order to exclude the possibility that these inhibitors may affect the membrane transport activity in Tetrahymena which might account for lower incorporation of ^{3}H-TdR, we decided to perform the uptake experiment of the labelled TdR by the treated cells. It was found that EGTA and TFP separately did not effect the uptake of ^{3}H-TdR. However, when TFP and

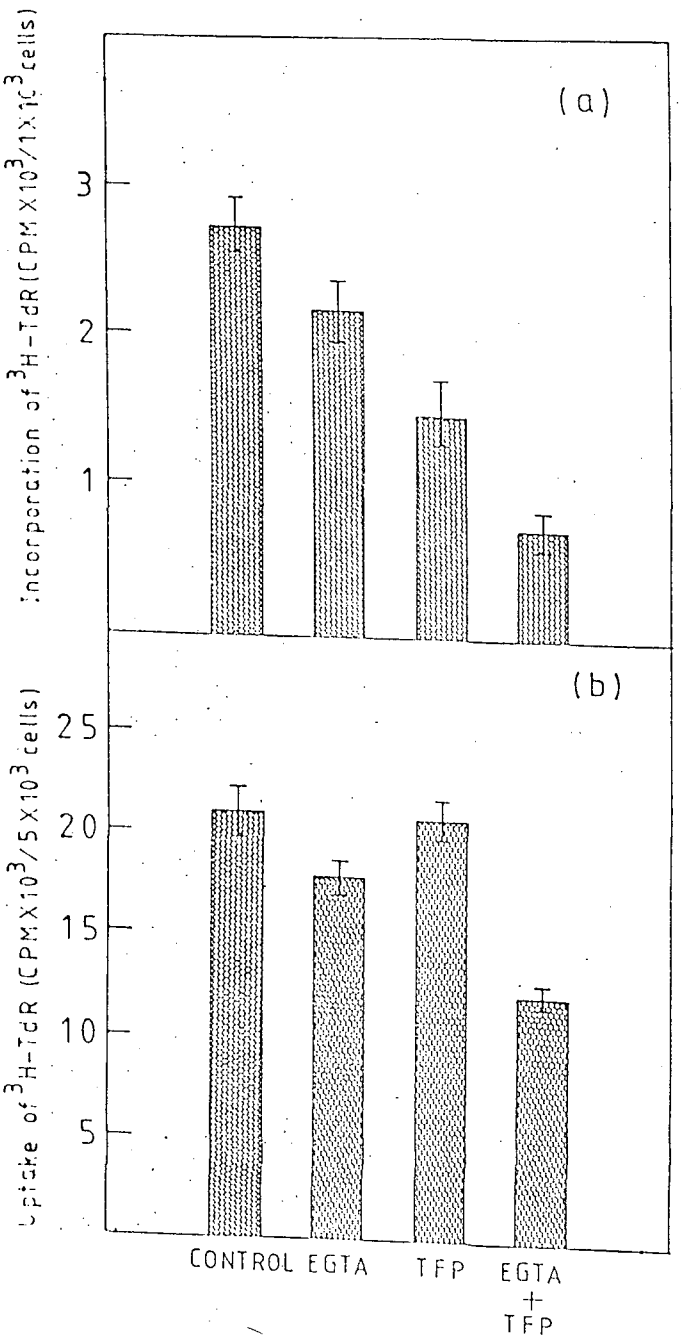

Fig. 12. a) ^{3}H-TdR incorporation profile in control Tetrahymena and in Tetrahymena treated with inhibitors of Calmodulin and Calcium at G_1/S boundary.

b) Uptake profile of ^{3}H-TdR in control Tetrahymena and in Tetrahymena treated with inhibitors of Calmodulin and Calcium at G_1/S boundary.

EGTA were simultaneously administered there was an appreciable inhibition (about 45%) ^{3}H-TdR uptake as compared to the control cells (Fig. 12b).

c) The anticalmodulin drug TFP was also administered to the synchronous mitotic cell population of Tetrahymena pyriformis. It was found that when the drug was administered to the culture, 10 minutes before the onset of synchronous division, the mitotic synchrony was not greatly affected. The mitotic index was found to be approximately 86% in the drug-treated cells as compared to approximately 88% in the control cells.

DISCUSSION

Since Ca^{2+} is involved in regulation of diverse intracellular events and the activity of CaM which is receptor of Ca^{2+}, is controlled by changes in the intracellular free Ca^{2+} concentrations, variable levels of CaM during the cell's life cycle probably indicates an important regulatory mechanisms for specific Ca^{2+} mediated events.

Tetrahymena pyriformis can be synchronized by multiple repeated heat-shocks to obtain cell populations at specific stages of cell cycle. This provides an opportunity to look into the pattern of changes of CaM, if any, during the different phases of cell-cycle. Moreover, the cellular physiology of this organism is known in great detail, so that involvement of this regulatory protein can possibly be ascribed to certain cellular functions.

Our investigations of CaM levels during different phases of cell cycle in Tetrahymena showed that during the G_1 phase, CaM concentration was maintained at the lowest level as compared to other phases of the cell-cycle. When the G_1 phase cells entered S phase, the CaM concentration was found to increase to about two folds, and this level was maintained

throughout G_2 and M phases. These observations are consistent with findings of Chafouleas et al (1982). They have reported that CaM levels are elevated two folds at late G_1 and/or early S during the growth cycle of CHO (Chinese Hamster ovary)-K_1 cells. It has also been shown that the intracellular levels of CaM are elevated at least two folds in all exponentially growing transformed cells regardless of the means of transformation (i.e. oncogenic viruses, chemical carcinogen or hormones etc.) as compared to non-transforming cells (Chafouleas et al, 1981; Means et al, 1982).

To test the hypothesis that CaM is involved in initiation of DNA synthesis and mitosis, we monitored the DNA synthesis (as measured by ^{3}H-TdR incorporation into nuclear DNA) in the cells treated with CaM and Ca^{2+} inhibitors at G_1/S boundary. Our studies demonstrated a drastic inhibition in the DNA synthesis in the TFP treated cells. When both TFP and EGTA were administered further suppression of DNA synthesis was noted, presumably, due to the additive effects of the inhibitors. However, our studies indicate that EGTA alone does not alter the process of DNA replication in any significant way.

That the membranes of the inhibitor treated cells were not affected in its transport properties was tested in our ^{3}H-TdR uptake studies. It has been seen that neither TFP nor EGTA can significantly prevent the transport of nucleotides in Tetrahymena cells. However, the simultaneous addition of both EGTA and TFP caused considerable reduction in uptake activity. This observation could be explained by a possible perturbation in the membrane structure which might occur due to severe depletion in membrane Ca^{2+} and its binding protein.

Elevation of the intracellular CaM concentration from the early G_1 level to the final 2X level of early S may be important for progression of cells into and through S phase. This concept of a critical concentration of CaM is compatible with the hypothesis of Mitchison (1974) that the progression from G_1 into S depends on the G_1 accumulation of a key substance to a critical threshold concentration.

Hazelton et al (1979) and Tupper et al (1980) have evaluated calcium sensitivity of late G_1 and have demonstrated that Ca^{2+} is required in late G_1

for progression into S phase to occur. The period just prior to S i.e. G_1 is the most variable period of the cell-cycle and can, in most cases, account for the different cell types. It is during G_1 that the commitment is made either to enter into S phase and continue cell proliferation or to exit from the cell cycle and enter into a quiescent phase (Mitchison, 1976; Prescott, 1976). Considering these observations it can be argued that CaM may be involved in this process of commitment.

Chafouleas et al(1984) have recently determined the levels of CaM mRNA quantitated by hybridization with a cloned human ^{32}P-CaM cDNA using a dot-blot procedure (White and Banecroft, 1982) and found that CaM-mRNA in CHO-K_1 cells decreased by 50% within 1 hour following the mitotic stimulus, but increased to control levels prior to the increase in CaM levels. These findings suggest that the changes in CaM levels are result of changes in CaM-mRNA. It can be speculated that the half-life of CaM is coupled to the half-life of CaM mRNA.

The intracellular distribution of and the cellular content of CaM in relation to nuclear activity

have been reported for animal cells (Welsh et al, 1979; Sasaki and Hidaka, 1982). Recently CaM-like activity associated with chromatin from pea-buds has been reported(Matsumoto, et al, 1983). These observations are very significant and that it may have some important function for the replication of DNA.

Ca^{2+} in partnership with cAMP controls the proliferation of non-tumorogenic cells in vitro and in vivo. While it does not seem to be involved in proliferative activation of cells such as hepatocytes (in vivo)on small lymphocytes (in vitro), it does control two later stages of pre-replication (G_1) development. It must be one of the many regulatory and permissive factors affecting early pre-replicative development, because severe calcium deprivation reversibly arrests some types of cell, in early G_1 phase of their growth-division cycle in vitro. However, calcium more specifically and much more often regulates a later (mid or late G_1) stage of pre-replicative development (Whitfield et al, 1979).

Thus regardless of its severity or the type of cell, Ca^{2+} deprivation in vivo and in vitro reversibly stops proliferative development at that part

of the G_1 phase in which the cellular cAMP content transiently rises and the synthesis of four deoxyribonucleiotides begins. This evidence points to Ca^{2+} and other cAMP surge being cogenerators of the signal committing the cell to DNA synthesis. This can be explained by the cAMP surge causing a rise of Ca^{2+} which combine with molecules of CaM, somewhere between the cell-surface and the cytosol. The resulting Ca^{2+} CaM complexes then stimulate many different (and possibly membrane associated) enzymes such as protein kinases, one of which produces the DNA synthesis initiator (Whitfield et al,1979). Finally, in this context, it might be relevant to point out here that Ca^{2+} contents in heat shock synchronized Tetrahymena population was measured by Walker and Zeuthen (1980). A significant peak of Ca^{2+} was detected just prior to division of the ciliates. However, according to these authors this increase probably indicate an effect rather than a cause.

During G_1/S transition period, several other proteins have been observed to increase in concentration. CaM has been shown to stimulate a variety of CaM-dependent protein kinases such as myosine light chain kinase and phosphorylase kinase. It is indirectly

involved in the activity of a large variety of protein kinase, through regulation of intracellular levels of Ca^{2+}, cAMP and cGMP. Cohen (1979) and Wolff et al (1981) have reported the activation of Ca^{2+}dependent phosphatases. So, it can be argued that CaM may be involved in regulating the cellular events by protein kinase and phosphatase activity to create a situation which can trigger the initiation of DNA synthesis.

Recent experimental results suggest that the increase in CaM level at G_1/S is important for optimal DNA repair prior to replicative DNA synthesis. If CaM is involved in DNA repair then some of the enzymes involved in purine and/or pyrimidine metabolism (or other proteins involved in DNA repair) must be regulated by CaM. It is already known that such enzymes are induced at G_1/S boundary. Hence, the possibility of involvement of CaM binding proteins cannot be ruled out (Means and Chafouleas, 1983).

It has been reported that coincident with entry into S the levels of atleast three of the Ca^{2+} dependent and three of the Ca^{2+} independent CaM

binding proteins increase. It can be suggested that increase in the Ca^{2+} dependent CaM binding proteins may be important for the initiation and/or progression of the cells through S (Glenney and Weber, 1980).

Another G_1/S boundary event which is very important is the phosphorylation of histones and non-histone chromosomal proteins which exhibit cell-cycle specific patterns (DeMorales et al, 1974; Bombik and Baserga, 1976; Costa et al, 1976; Sons et al, 1976; Gurley et al, 1978). Phosphorylation of histones H_1 and non-histone chromosomal proteins increase in late G_1 and early S phases. While the subsequent pattern of H_1 phosphorylation throughout the cell-cycle is complex, phosphorylation at the G_1/S boundary appears to be an important signal in the cell.

Crossin and Carney (1981) have suggested that microtubule depolymerization is involved to mediated the Ca^{2+}- directed depolymerization of microtubules in vitro (Marcum et al, 1978; Nishida et al, 1979; Jemiolo et al, 1980; Job et al, 1981). Since this regulation depends on the CaM concentration relative to that of tubulin, elevation of CaM concentration should result in a shift towards a greater proportion

of depolymerized tubulin in the cell. Such an event would be predicted to occur at the G_1/S boundary where CaM levels are increasing. Chafouleas et al (1981) have suggested that CaM may regulate the cytoplasmic microtubule network in a direct manner through regulation of tubulin synthesis.

In conclusion, our findings strongly suggest that CaM has an important functional role in nuclear DNA replication process in the cell. This is consistent with the results of MacManus et al, 1975 and Boyton et al 1980, who have also made similar investigations. The exact mechanism by how CaM interacts in DNA synthetic process is yet to be unrevelled and awaits further investigations on this aspect of calmodulin activity.

Our experiments on the effect of CaM anatagonist on the synchronous mitotic divisions have failed to reveal any possible involvement of CaM in the cell-division process. But our limited experimental approach cannot preclude the possibility of CaM having a significant functional role in cell-division process. The presence of CaM in the nucleus of mitotic cells have been clearly demonstrated (Welsh et al, 1979;

Zavotinsk et al, 1983) which strongly indicates a possible functional role for these molecules during cell division. The exact interrelationship between CaM and components of the mitotic apparatus has yet to be established by further experiements.

SUMMARY

1. Calmodulin (CaM) is a heat stable, low molecular weight, acidic, Calcium-binding protein in eukaryotic cells. The level of CaM in different phases of cell-cycle of Tetrahymena pyriformis was determined.

2. To determine the level of CaM in different phases of cell-cycle, Tetrahymena cells were synchronized in the mitotic phase by multiple repeated heat shock method.

3. The duration of the phases of cell-cycle was determined by pulse labelling the cells with ^{3}H-TdR and it was found that cells exhibit a G_1 of 60 minutes; S of 40 minutes; G_2 of 60 minutes and M of 20 minutes.

4. The partially purified homogenates of Tetrahymena from different phases of cell-cycle were subjected to electrophoresis on SDS-polyacrylamide gels with bovine brain CaM as standard.

5. The relative levels of CaM were quantitated by densitometric scanning of gels. It was found that the concentration of CaM in the mid-G_1 was approximately half of the concentration of S and this $2XG_1$ level of CaM was maintained through G_2 and M.

6. To find out any possible role of CaM in the initiation of DNA synthesis, the incorporation of ^{3}H-TdR experiment was performed at G_1/S boundary in the presence of EGTA (Ca^{2+} chelator) and TFP (CaM antagonist) and also in simultaneously presence of EGTA +TFP. It was found that these inhibitors were able to inhibit the DNA synthesis in the additive manner.

7. In order to exclude the possibility of alteration of membrane transport properties in the presence of EGTA and TFP, the uptake experiment was performed. It showed that individually the inhibitors did not affect the uptake of ^{3}H-TdR, although the simultaneous application of EGTA and TFP showed the suppression of ^{3}H-TdR uptake.

8. The administration of TFP, before the start of mitotic division of cells, could not significantly change the progression through M phase.

9. Our experimental results demonstrated the variable content of CaM throughout the cell-cycle of Tetrahymena pyriformis and strongly suggested the possible involvement of this Ca^{2+}- binding protein in nuclear DNA replication process.

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