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CALMODULIN AND THE ASSEMBLY AND DISASSEMBLY OF MICROTUBULES

DURING THE MITOGENIC STIMULATION OF MOUSE T

LYMPHOCYTES

by

Roberto Campos González

A thesis submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the Department
of Biology in the School of Graduate Studies and
Research of the University of
Ottawa

Spring 1987



Roberto Campos Gonzalez, Ottawa, Canada, 1987.

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To my wife, Mary Tere, my daughter, Maria Teresa
and my son, Roberto Arturo

To my parents, Maria del Refugio and Roberto

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ABSTRACT

The purpose of my research was to examine the subcellular localization of calmodulin and its relationship to the dynamic changes of the microtubule system during the mitogenic stimulation of mouse T lymphocytes.

Double immunofluorescence staining, with affinity purified antibodies to calmodulin and monoclonal antibodies to tubulin, was used to study temporal and spatial relationships between calmodulin and microtubules under different experimental conditions. Immunoblotting showed that the two antibodies reacted only with their respective antigens in different tissues and cell types, including mouse T lymphocytes. Immunofluorescence staining of Triton X-100 permeabilized cells revealed a diffuse localization of calmodulin in the cytoplasm of resting T lymphocytes and of lymphocytes stimulated for 24 hours with the mitogen concanavalin A. In fully stimulated lymphocytes, observed after 48 hours of culture with mitogen, calmodulin was localized to the centrosome and to some microtubules.

The localization of calmodulin to the centrosomal region correlated with the increase in microtubules assembled from the centrosome that occurs during stimulation. The calmodulin-centrosome interaction remained throughout mitosis, with calmodulin localized to the half spindles during metaphase and to the interzone microtubules

in late anaphase and telophase. The localization of calmodulin to the centrosome to fully stimulated lymphocytes was resistant to permeabilizations in the presence of 10 mM EGTA, therefore indicating that the interaction is calcium-interdependent. Permeabilization of cells in the presence of 1.2 mM CaCl_2 resulted in the disassembly of most interphase and mitotic microtubules; however, some kinetochore microtubules were resistant to the calcium exposure, and by immunofluorescence were calmodulin positive. Treatment of stimulated lymphocytes with colchicine or colcemid resulted in microtubule disassembly, but did not alter the localization of calmodulin to the centrosome. Exposure of stimulated lymphocytes to low temperature, on the other hand, resulted in both disassembly of microtubules and a disruption of the calmodulin-centrosome interaction. During recovery from low temperature, calmodulin relocated to the centrosome coincident with microtubule reassembly. Taxol promoted microtubule assembly and the formation of bundles of microtubule reassembly. Taxol promoted microtubule assembly and the formation of bundles of microtubules from the centrosome. Calmodulin colocalized to the taxol-induced microtubule bundles. These taxol-induced and calmodulin-containing microtubules were resistant to permeabilization in the presence of 1.2 mM CaCl_2 . Low doses of calmodulin antagonists (naphthalenesulfonamides, trifluoperazine and

calmidazolium) did not significantly disturb the normal microtubular pattern in the fully stimulated lymphocyte, nor did they affect cell viability. However, the same doses of the antagonists, but not ineffective analogs, blocked the reassembly of microtubules during recovery from cold treatment.

Brij-58 permeabilized stimulated lymphocytes and 3T3 cells were exposed to increasing concentrations of Ca^{2+} . The cells showed a remarkable stability of their microtubules up to 50 μM calcium. Exposure of the cells to 0.1 mM or 1.0 mM calcium did cause the disassembly of interphase microtubules; however, microtubules close to the centrosomes and most mitotic microtubules remained intact. Calmodulin antagonists did not prevent the calcium-induced disassembly of microtubules; however, 10 μM of the calpain inhibitor leupeptin did block the disassembly. The same doses of PMSF or an excess of soybean trypsin inhibitor were ineffective. These results have suggested that the calcium-induced disassembly of microtubules is mediated by a calcium-dependent proteolytic mechanism. Cellular tubulin did not appear to be the target for the calcium-dependent proteolysis.

Together, these results indicate the existence of a calmodulin-centrosome interaction which is calcium-independent, colchicine and colcemid resistant, and cold-labile. Calmodulin interacts with centrosomes only during

an increased capacity of the latter to assembly microtubules. Calmodulin activity also appears to be required during the reassembly of microtubules following cold treatment. Calmodulin is not involved in the calcium-induced disassembly of microtubules. A calcium-dependent proteolytic event may mediate the calcium effects on microtubules.

RESUME

Mon travail de recherche avait pour but d'examiner la localisation subcellulaire de la calmoduline et sa relation avec les changements dynamiques du système de microtubules pendant la stimulation mitogénique des lymphocytes.

Un double marquage des cellules par immunofluorescence, avec des anticorps purifiés pour leur affinité avec la calmoduline, et des anticorps contre la tubuline, a été utilisé pour étudier la relation spatiotemporelle entre la calmoduline et les microtubules dans différentes conditions expérimentales. L'utilisation de la technique d'immuno-absorbance a permis de montrer que les deux anticorps réagissaient uniquement avec leur antigènes respectifs dans différents tissus et types de cellules incluant les lymphocytes T de souris. La coloration par immunofluorescence de cellules rendues perméables par le Triton X-100 a révélé une localisation diffuse de la calmoduline dans le cytoplasme des lymphocytes T au repos et dans les lymphocytes stimulés pendant 24 heures avec un agent mitogène, la concanavaline A. Dans les lymphocytes complètement stimulés observés après 48 heures de culture en présence du mitogène, la calmoduline était localisée au niveau du centrosome et de quelques microtubules.

La localisation de la calmoduline dans la région du centrosome coïncidait avec l'accroissement de l'assemblage de microtubules à partir du centrosome qui a lieu pendant la stimulation. L'association calmoduline-centrosome était maintenue pendant la mitose et la calmoduline était aussi localisée au niveau du fuseau (half spindle) pendant la métaphase et dans l'"inter-zone" en anaphase tardive et en télophase. Dans les lymphocytes complètement stimulés, la présence de calmoduline au niveau du centrosome n'était pas affectée par la présence de 10 mM EGTA au moment où les cellules étaient rendues perméables, ce qui indique que l'interaction entre la calmoduline et le centrosome est calcium-indépendante. L'augmentation de la perméabilité cellulaire en présence de 1.2 mM CaCl_2 a entraîné le désassemblage de la plupart des microtubules interphasiques et mitotiques. Cependant, quelques microtubules attachées aux kinétochores étaient résistantes à la présence de calcium, et une coloration par immunofluorescence a montré la présence de calmoduline à leur niveau.

Le traitement des lymphocytes stimulés avec de la colchicine ou de la colcémide a entraîné par ailleurs la dissociation des microtubules et la disparition de l'interaction entre la calmoduline et le centrosome. Pendant la récupération du traitement à basse température, la relocalisation de la calmoduline au niveau du centrosome coïncidait avec la repolymérisation des microtubules. Le

taxol augmentait l'assemblage des microtubules et la formation de groupes de microtubules à partir du centrosome. La calmoduline se retrouvait associée aux groupes de microtubules assemblés en présence de taxol. Ces microtubules qui ont polymérisé en présence de taxol et qui contiennent de la calmoduline étaient résistantes à un traitement avec 1.2 mM CaCl_2 . De faibles doses d'antagonistes du calcium (naphtalenesulfonamides, trifluoperazine et calmidazolium) n'ont pas eu d'effets significatifs sur la distribution des microtubules dans les lymphocytes stimulés et n'ont pas affecté la viabilité cellulaire. Les mêmes doses d'antagonistes ont bloqué la repolymerisation des microtubules pendant la récupération des cellules après un traitement au froid.

Des lymphocytes stimulés rendues perméables avec le Brij-58 et des cellules 3T3 ont été exposés à différentes concentrations de Ca^{2+} . Les microtubules étaient relativement stables à des concentrations allant jusqu'à 50 μM Ca^{2+} . Un traitement des cellules avec des concentrations de 0.1 mM ou de 1.0 mM Ca^{2+} a entraîné la dissociation des microtubules dans les cellules en interphase. Les microtubules près des centrosomes et la plupart des microtubules des cellules en mitose n'ont pas été affectées par le traitement. Les antagonistes du calcium n'ont pas empêché le processus de dissociation des microtubules par le calcium; cependant, un traitement avec

10 μ M de leupeptin un inhibiteur de "calpain" a bloqué le désassemblage des microtubules normalement induit par le calcium. La même dose de PMSF ou un excès d'inhibiteur de la trypsine de fève soya n'ont eu aucun effet. Ces résultats suggèrent que le désassemblage des microtubules induit par le calcium soit due à un mécanisme protéolytique qui est dépendant du calcium. La tubuline cellulaire ne semblait pas être la cible de la protéolyse due à la présence de calcium.

Les résultats obtenus indiquent l'existence d'une interaction entre la calmoduline et le centrosome qui n'est pas dépendante de la concentration de calcium qui est résistante à la colchicine et à la colicémide et qui est sensible à un traitement au froid. Il y a interaction entre les centrosomes et la calmoduline uniquement au moment où il y a une augmentation de la polymérisation de microtubules au niveau du centrosome. Il semble que la calmoduline est aussi requise lors de la polymérisation des microtubules après un traitement au froid. La calmoduline n'est cependant pas impliquée dans la dépolymérisation des microtubules entraînée par la présence de calcium. Une substance protéolytique calcium-dépendante agissant sur des protéines microtubulaires autres que la tubuline pourrait servir de médiateur dans l'effet du Ca^{2+} sur les microtubules.



INTRODUCTION

The interest in the roles of calcium in living systems has increased dramatically in recent years. Today, it is widely accepted that the calcium ion triggers and regulates such diverse aspects of cellular activity as secretion, excitability, division and motility. Unlike other ions, calcium was adopted through evolution as a regulator (1). For instance, an increase in the intracellular calcium concentration is the mediator between an external stimulus and the contractile activity of muscle cells.

The organization of the cytoskeleton has attracted the attention of investigators during the last twenty years. In particular, the microtubular system has been shown convincingly to influence many aspects of cellular function. The activity of the microtubules (MT) depends on a dynamic assembly of their components that lead to a specific cellular response (2). The discovery of Weisenberg (3) that reduced calcium concentrations promoted the assembly of brain MT in vitro led to the suggestion that calcium and calcium-binding proteins (CaBP) like calmodulin (CaM) have a physiological role in the regulation of assembly and disassembly of MT in the cell.

Despite the many recent reports proposing calcium and calmodulin as physiological regulators of MT equilibrium, no conclusive evidence is yet available. Questions still remain

concerning the mechanism of calcium and calmodulin action. How does calcicalmodulin regulate the assembly and disassembly of MT? Does the intracellular localization of calcicalmodulin correlate with the assembly or disassembly of MT? Do all MT have the same sensitivity to calcium? What is the microtubular component that mediates the calcicalmodulin effects?

In recent years it has become evident that the mitogenic stimulation of mouse T lymphocytes induces an increase in the number of microtubules and changes in the organization of the centrosome of those cells (4). The dynamic characteristic of the microtubular system in mouse T lymphocytes during stimulation, therefore, makes these cells a suitable model for the study of the relationship between the CaM distribution and the changes of the microtubular system. In this thesis, I propose that the study of the subcellular distribution of CaM in the mitogen stimulated mouse T lymphocyte should provide insights into the CaM-microtubular system interaction.

Microtubules

It is now very well established that the interior of the eukaryotic cell has a high level of organization. The nucleus and other organelles have defined positions between

the limits of the plasma membrane. Cellular organization is required for the optimal functioning of the living cell.

In recent years the intracellular filamentous systems, collectively named "the cytoplasmic matrix", have been implicated in the maintenance of the intracellular order (5). Microtubules form one of those filamentous systems and are found in ciliary and flagellar axonemes, mitotic spindles and the cytoplasm of eukaryotic cells. Microtubules are very dynamic structures that can assemble and disassemble according to the physiological state of the cell, and they are now accepted as essential elements in such diverse cellular functions as motility, secretion, intracellular transport and division (for a general review see 2).

Structure and composition.

Microtubules are hollow cylinders of variable length and with a diameter of about 24 nm. The wall of the microtubule is made of thirteen protofilaments running longitudinally. The protofilament is formed by self assembly of protein subunits, and each subunit is an heterodimer of alpha and beta tubulin. Each has an Mr (relative mass) of approximately 55 kD (kilodaltons). In cross section, each microtubule consists of thirteen globular structures of four to five nm in diameter. Each globular structure corresponds to an heterodimer of alpha and beta tubulin.

In addition to tubulin, microtubules also have other components named microtubule-associated proteins (MAPs). The types of MAPs in MT may vary according to the cell type and with the specific developmental stage of a particular cell or organism (6). - The most extensively characterized MAPs have been obtained from mammalian brain tissue. They are the MAP1 (Mr: 350 kD), MAP2 (Mr: 270 kD) and tau proteins (Mr: 55-70 kD). These proteins co-purify with tubulin in stoichiometric amounts during repeated cycles of microtubule assembly and disassembly. MAPs 1 and 2 have been localized within cells as lateral projections from the outside wall of the MT (7 and 8).

In vitro assembly.

In vitro studies have elucidated the basic characteristics of MT assembly and have examined the effects of individual cellular components on the assembly and disassembly of MT. These studies have indicated that MT assembly has the characteristics of a nucleated condensation polymerization process (9). The assembly of MT in vitro occurs in several steps: a) the initiation of nucleation; b) the formation of the nuclei; and c) the elongation of the polymer. The formation of the nuclei requires the appearance of protofilament fragments which associate laterally (10). Elongation occurs by the addition of subunits at the ends of

the protofilaments. Optimal in vitro assembly of MT requires a minimum concentration of tubulin (approximately 1.0 mg/ml), μ M concentrations of calcium, mM concentrations of magnesium, guanosine nucleotides, temperatures of about 37°C and a pH around 6.9.

In vivo assembly and disassembly.

The ability of MT to assemble and disassemble according to the physiological state of the cell is its most remarkable property. For example, cells disassemble their cytoplasmic microtubules when entering mitosis, and the opposite occurs when daughter cells separate after telophase. Recent observations of living cells suggest that most MT are not static, but rather are in a continuous assembly and disassembly process. For example, it has been shown that the turnover of cytoplasmic MT in BSC1 cells is about 10 to 15 min (11).

In living cells, most MT grow from defined intracellular localities named microtubule-organizing centers (MTOC) such as centrosomes, polar plaque, basal bodies, etc. (for a general review see 12). Centrosomes are composed of a pair of centrioles surrounded by an osmiophilic material termed the pericentriolar material, which includes the satellite bodies. The pericentriolar material undergoes changes in morphology according to the stage of the cell cycle. For

example, satellite bodies of interphase cells become disorganized and appear as an amorphous pericentriolar material when the cells enter mitosis (13).

The use of limited cell permeabilization has allowed a more direct manipulation of the assembly of MT from the centrosome (14). Essentially, cells with disassembled MT are permeabilized with a detergent. The permeabilized cells are then incubated with exogenous tubulin in a variety of conditions and in the presence of several factors like GTP, Mg^{2+} , etc. With this procedure, we now know that the ultrastructural changes of the centrosome during the cell cycle are usually accompanied by changes in the capacity of the centrosome to nucleate and assemble MT. For example, the assembly capacity of the pericentriolar material in some cultured cells increases as the cells enter mitosis (15) and decreases again during the metaphase/anaphase transition (16). The current hypothesis is that centrosomes may regulate the temporal assembly of MT (12).

MTOCs, like the centrosome of mammalian cells, may not be merely a site of the nucleation of MT. Centrosomes might also regulate the number of protofilaments and ultimately the number of MT within the cell. Centrosomes might also exert a regulatory function on the spatial nucleation and distribution of MT (12, 13).

Microtubule-associated proteins in the assembly of MT.

MAPs might be the physiological regulators of MT dynamics. They co-purify with tubulin, they have also been localized along MT in a variety of cell types, and MAPs promote the assembly and stabilize MT in vitro (8). However, the exact mechanism of action of MAPs is still unknown. MAP2 can be phosphorylated by cAMP-dependent and calcineurin-dependent protein kinases. Upon phosphorylation, the ability of MAP2 to promote the assembly of MT is reduced (17). MAP2 can also be proteolyzed in vitro by calcium-activated proteases, which impair the ability of MAP2 to promote MT assembly in vitro (18). It remains to be established how phosphorylation, proteolysis or other covalent modification of MAPs (and tubulin) affects the dynamics of MT in vivo (19).

Calcium

The extracellular concentration of calcium and the free calcium (Ca^{2+}) concentration in the cytoplasm are important regulators of cellular functions such as nerve excitability, clot formation, muscle contraction, secretion and proliferation (20).

Within the cell, small pulses of free calcium occur as a response to diverse physiological conditions such as hormone-

binding to the plasma membrane, fertilization and membrane depolarization (1). It is believed that the small increases of free calcium act as a second messenger of the information carried by the inducer or first messenger. The calcium source responsible for the increases is different according to the cell type, but it is either an influx from the extracellular medium or an efflux of calcium stored in membranous systems, such as sarcoplasmic reticulum in muscle cells, endoplasmic reticulum in non-muscle cells (21), and the series of vesicles around the mitotic poles in dividing cells (22).

Recent technical advances have allowed the monitoring of changes in intracellular calcium concentrations in individual cells. The new series of non-toxic synthetic fluorescent calcium chelators (quin-2, fura 2, TPEN) with a sensitivity of about $10^{-7}M$ (23, 24) are polycarboxylate anions that can be incorporated into the cell if the carboxylate groups are esterified. Once the indicator is inside the cell, it is converted to the original compound by cytoplasmic esterases. The loaded cells are then analyzed fluorometrically or by a combination of image processing and fluorescence microscopy.

These techniques have been useful in determining the intracellular free calcium concentration in several cell types during different stages of the cell cycle. For example, it was found that PtK2 cells had a calcium concentration of 52 nM during interphase and, as they passed

throughout mitosis, decreased their intracellular calcium concentration to 26 nM (25). Calcium concentration has also been measured during fertilization in individual eggs of the sea urchin Lytechinus. In these cells, there are submicromolar increases of calcium at fertilization, during pronuclear migration, nuclear envelope breakdown, metaphase/anaphase transition and cleavage (26).

The current hypothesis for the mode of action of calcium (see 21) proposes that the calcium signal carried by the changes in calcium concentrations, transfers its information to specific calcium acceptors such as CaM and troponin C. These proteins then modulate the activity of several different enzymes.

Calcium-binding proteins.

The identification of intracellular calcium acceptors is an essential step in an understanding of calcium action within the cell. Eukaryotic cells have a family of similar, low molecular weight, intracellular calcium-binding proteins. Calmodulin belongs to this family of CaBP. The amino acid sequence homology among those heat-stable proteins immediately suggested that they are genetically-related. Possibly they arose through gene duplications from an original gene coding for a single calcium-binding domain (27).

The family of CaBP has affinities for calcium in the range of 10^{-6} to 10^{-5} M at physiological pH, ionic strength and magnesium concentrations (28). Studies pioneered by Robert Kretsinger, who analyzed the structure of carp parvalbumin (29), suggested that all members of this CaBP family bind calcium in specific domains formed by acidic and hydroxylated residues. This "calcium pocket" has the oxygen atoms from the amino acid donors precisely arranged to accommodate the calcium ion with the help of a water molecule. The number of calcium-binding sites is variable for each protein of this family. Each calcium-binding site is flanked by two short alpha-helical structures oriented at 90° to each other (29). The entire domain of alpha-helix:loop:alpha-helix is named the "EF hand" (E and F are the last two helical regions in parvalbumin).

There are at least two major functions proposed for the calcium-binding proteins. First, they may become part of an intracellular "calcium sink" that prevents the calcium concentration from rising to undesirable or harmful levels. Examples of this proposed role are the parvalbumins in muscle and the vitamin D-dependent CaBP in the intestinal brush border (28). A second role for some of the CaBP depends upon their ability to change their conformations in the presence of calcium. Thus, only the calcium-CaBP complex is able to induce changes in the activity and conformation of target proteins. The best known example is troponin C. Calcium-

binding to troponin C, changes the structure of the troponin complex, which removes tropomyosin from myosin, thus allowing the interaction of actin and myosin, which results in the contraction of the muscle fiber (30).

Calmodulin

Calmodulin changes the activity of a wide variety of enzymes and processes in the eukaryotic cell (for a general review see 31). Calmodulin was discovered sixteen years ago as an activator of cyclic nucleotide phosphodiesterase (PDE) from mammalian brain. Since then, CaM has been found in every eukaryotic organism examined and is a highly conserved protein. For example, only twelve conservative amino acid substitutions exist between human and Tetrahymena calmodulins (32).

Calmodulin binds four moles of calcium per mole of protein with a $K_d = 2 \times 10^{-6} M$. The K_d is further decreased by an order of magnitude when CaM binds to its target proteins (33). Calmodulin is about 0.5% to 2% of the total soluble proteins, and it has been proposed as a major intracellular calcium acceptor (21).

Table I lists some of the physiochemical properties of vertebrate calmodulin. This protein has 148 amino acids, of which approximately one-third are of an acidic nature. Calmodulin does not contain cysteine, tryptophan or hydro-

TABLE I. Physicochemical properties of vertebrate calmodulin.

Molecular weight	16 700 (148 amino acids)
Heat resistance	$t_{1/2} = 7$ minutes at 100°C
Sedimentation constant $S^{0}_{20,w}$	1.9
Phe/Tyr	4/1
f/f_0^*	1.34
E 275-278, 1% 1 cm	2.1
Isoelectric pH	3.9
Trimethyllysine	Lysine 115
% Acidic residues (Glx + Asx)	25%
Calcium-binding	4 mol/mol; $K_d = 2.4 \mu\text{M}$
Conformation	40% - 55% α -helix
Sequence homology	50% to troponin C
Drug-binding	Phenothiazines, W compounds
Hydrophobicity	Increased by calcium

Reference 31.

xyproline, but does contain the unusual trimethyllysine. Figure 1 illustrates both a schematic model of CaM (Fig. 1a), which includes its amino acid sequence, and the proposed model for CaM deduced from X-ray crystallography (Fig. 1b). According to Babu et al. (34), who recently described the three-dimensional structure of calmodulin with a resolution of 0.30 nm, the molecule has two globular ends connected through a long stretch of alpha-helix (Fig. 1b). Each end consists of two calcium-binding domains.

Nuclear Magnetic Resonance studies of the NH₂ and COOH terminal halves of CaM have indicated that these fragments still retain several of the properties that they have in the native molecule. These properties include calcium-binding and the ability to interact with phenothiazines (35). It has also been suggested that calcium-binding sites III and IV (in the COOH terminal) have higher affinity for calcium than sites I and II (36). Thus, the calcium-binding by calmodulin may happen in a step-wise manner that results in at least three different conformers: CaM, Ca₂²⁺ - CaM and Ca₄²⁺ + CaM.

Several groups have now been successful in elucidating the basic features of the CaM gene. Simmen et al. (37) cloned and sequenced the chicken CaM gene. They found that this particular gene has eight exons and seven introns. Interestingly, the distribution of the introns suggests that they are located with no relation to the calcium-binding

Figure 1. Model of Calmodulin.

a) Schematic model of CaM illustrating the four calcium-binding sites and the primary structure.

b) The proposed structure of CaM deduced from X-ray crystallography according to Babu et al.

(34). One pair of calcium-binding sites is located at each end of the molecule. I and II are located at the NH_2 terminal. III and IV are located at the COOH terminal.

domains of the protein. The CaM gene in Xenopus eggs also appears to have several introns (38). It has also been found that Trypanosome has several copies of the CaM gene (39). Molecular biology techniques may be useful in the study of calmodulin; for example, molecular variants of CaM may become available through site-directed mutagenesis. Those molecular variants of CaM will be invaluable in the study of the mode of action of calmodulin.

Mode of action of calmodulin.

Calmodulin modifies the activity of several enzymes in a calcium-dependent manner. It also affects several cellular processes in which the calmodulin-dependent enzymes are involved. Because of the multiple CaMBP existent within the cell, calmodulin may affect several cellular functions simultaneously. For example, calmodulin localized in the plasma membrane regulates the rate of calcium efflux, while simultaneously CaM localized in the cytoplasm might modulate the rate of glycogen breakdown.

Although the binding of calcium to calmodulin is an essential feature for the activity of CaM, questions still remain with regard to the exact mechanism of CaM interaction with its binding proteins. Apparently, the binding of calcium exposes a hydrophobic domain in CaM (40) that appears to be a major site for interaction with CaMBP. Calmodulin

also can interact with several enzymes and other proteins in the absence of calcium. Such is the case for adenylate cyclase from Bordetella pertussis (41) and phosphorylase kinase (42); however, the full activation of the two enzymes still requires calcium. The 110 kD CaMBP of intestinal brush border also interacts with CaM and with actin in the absence of calcium (43). It had been suggested that calcium-independent interactions between CaM and its binding proteins increase the affinity of CaM for calcium (33), and therefore this mechanism possibly increases the CaM sensitivity to small changes of intracellular calcium concentrations.

Chemical approaches have also been useful in the attempt to understand the mode of CaM action. Selective modifications and cleavage of the CaM molecule have shown that CaMBP may recognize discrete domains of CaM. For instance, carboxymethylation of methionine residues 71, 72 and 74 of CaM block its interaction with PDE (44). Also Ni and Klee (45) have shown that residues 1 to 77 were able to interact with PDE and cAMP-dependent protein kinases, whereas residues 78 to 148 were found more specific for myosin light chain kinase and calcineurin.

Post-transcriptional modifications also seem to modulate CaM activity. The de-methylation of trimethyllysine 115 of CaM made this molecule a more potent activator of NAD kinase without affecting its properties as an activator of PDE (46).

Finally, it is well known that CaM redistributions occur during the cell cycle and in response to specific stimuli. The best known example is the striking shift of CaM from being diffusely distributed in interphase cells, to being localized in the spindle poles and kinetochore MT and cleavage furrow of mitotic cells (47, 48). Also, adrenocorticotrophic hormone induces a translocation of CaM from the cytoplasm to the nuclear membrane in adrenal cortex cells (49); dopamine releases CaM from membranes to cytoplasm in nervous tissue (50); gonadotrophic-releasing hormone induces a CaM redistribution from membranes to cytoplasm of pituitary cells (51); and the beta-adrenergic activation of rat parotid glands induces an accumulation of calmodulin in the cytoplasm (52). The significance of these CaM redistributions is at present not fully understood; however, they may represent a compartmentation mechanism for the regulation of calmodulin activity.

Calmodulin-binding proteins.

The precise nature of the calmodulin mode of action obviously requires the identification of CaM-binding proteins (CaMBP). Such studies have been done in recent years with a variety of techniques such as affinity chromatography, photoaffinity cross-linking, ^{125}I -calmodulin-binding in polyacrylamide gels, and most recently by biotinylated CaM-

binding to proteins immobilized on nitrocellulose. The number of CaMBP known increases every month, and ranges from cytoskeletal components to histones. Never can complete lists of CaMBP be found in the reviews by Manalan and Klee (53) and Klee and Vanaman (31). The molecular and kinetic studies of the interaction of CaM and its binding proteins have demonstrated that these CaMBP do not have a common CaM-binding subunit, nor do they have a similar CaM-binding domain (31).

Calmodulin also is able to interact with several small peptides such as endorphin, mellitin, mastoparans, etc. (54). The physiological importance of such interactions remains obscure. However, recent work indicates that the ability to form an amphipathic helix is an important requirement for those peptides to bind CaM (55). This feature was an important insight for the recent development of CaM-antagonists with K_d of $10^{-12}M$ (56).

Calmodulin antagonists.

A very useful approach to understanding the biological role of CaM has been the application of pharmacological compounds that interfere with the activation of enzymes by calmodulin in the presence of calcium (for a detailed list of CaM antagonists and recent reviews see 57). These antagonists, in vitro, show a wide range in affinity for

calcicalmodulin (K_d : 10^{-6} to $10^{-4}M$). The nature of the antagonists also shows a wide spectrum and includes some muscle relaxants such as naphthalenesulfonamides (W compounds), antipsychotics like trifluoperazine, and non-ionic detergents like Triton X-100.

Most of the antagonists share a minimum of similarity in their chemical structures. These CaM antagonists have two hydrophobic regions and at least one positively charged group attached to the hydrophobic groups by an alkyl chain. They interact with calmodulin only in the presence of calcium. These drugs have been used to purify calmodulin from a crude tissue extract (58) and also to study the calcium-calmodulin activation of enzymes in vitro.

Calmodulin has two phenothiazine-binding sites ($K_d = 10^{-6}M$). Phenothiazines can form covalent adducts with CaM, and they have recently been used in the study of enzyme activation by CaM. For example, Newton et al. (59) found that chlorpromazine-CaM resulted in the inhibition of PDE; however, the chlorpromazine-CaM also ~~was~~ a potent agonist for glycogen synthase and phosphorylase kinase.

In vivo studies are facilitated because the antagonists are very hydrophobic and therefore can easily penetrate the plasma membrane. However, it is known that high concentrations of antagonists can interact with proteins other than CaM and may even directly block their enzymatic activities (57). Therefore, a minimum number of criteria

should be met in order to assign an inhibition of CaM activity by the antagonists in vivo. For example, the active dose of the drugs should be close to their antagonistic activities in vitro. Non-active analogs, like dechlorinated naphthalenesulfonamide W-5 or trifluoperazine sulfoxide, must not block the cellular functions examined. The antagonist should block cellular processes in the presence of calcium.

Calmodulin and the cytoplasmic matrix.

It has become evident that CaM interacts with the cytoplasmic matrix of eukaryotic cells in a variety of ways. For instance, immunofluorescence studies and microinjection of labelled analogs of CaM have demonstrated an interaction with stress fibers in non-muscle cells (48, 60). Since CaM does not bind to actin, the actin-CaM interaction might be mediated by MAP2, which is able to interact with both actin and calmodulin.

It appears that calcium-calmodulin modulates the actin-myosin system by activating several key enzymes that are important for contractility (61). Myosin light chain kinase is a calcicalmodulin-dependent enzyme that catalyzes the transfer of Pi to the regulatory chains of myosin. Myosin kinase, another calcicalmodulin-dependent enzyme, directly phosphorylates the heavy chains of myosin. These phosphorylations promote the actin-myosin interactions and

activate the myosin ATPase, which provides the energy for contractile activity in non-muscle cells.

Calmodulin is also able to interact with several other actin-binding proteins such as caldesmon obtained from chicken gizzard, a 110 K dalton protein from intestinal epithelial cells, and spectrin and spectrin-like proteins (62). Calmodulin might also preserve the stability of actin filaments since TFP induces the loss of actin filaments in Hela and 3T3 cells (63). A recent report suggests that CaM by unknown mechanism, might increase the rate of actin polymerization in human platelets (64). An interaction of CaM with actin is further supported by studies of the actin filament system in the intestinal brush border. Here, CaM and the vit D-dependent CaBP keep a low intra-cellular calcium, thus protecting the actin filaments from the calcium-dependent and actin-severing activity of villin (43).

Calmodulin might also influence the intermediate filament system. For example, vimentin filaments are phosphorylated by a calcicalmodulin-dependent protein kinase (65). Desmocalcin, a 240 K dalton protein isolated from desmosomes of bovine muscle epidermis, is able to interact in vitro with CaM in a calcium-dependent fashion, as well as with keratin filaments (66).

Calmodulin and microtubules.

The involvement of CaM in the microtubular system is derived from two different areas of microtubule research. One is the study of ciliary and flagellar function. It is known that CaM is found in cilia and flagella from different cell types and organisms. Calmodulin and other CaBP are possibly involved in the regulation of ciliary beat initiation and frequency (67) and the asymmetry of flagellar bending (68). Calmodulin has been localized in the membrane, matrix and outerdoublet MT from cilia of Tetrahymena (69). Calmodulin has also been localized along the MT and dynein arms in cilia from the hamster tracheal epithelium (70) and in the ciliary basal bodies in several organisms (71). It has been suggested that calmodulin might modulate ciliary and flagellar function through the activation of the 14S dynein-ATPase (72).

As mentioned before, the assembly of brain MT in vitro is promoted if the excess of calcium is chelated by EGTA (3). It should be pointed out here that MT assembled in vitro from brain microtubular protein (tubulin + MAP) required 1.0 mM CaCl_2 to be disassembled; however, sensitivity to μM amounts of calcium could be achieved by the addition of a heat labile factor obtained from brain extract (73), which does not co-purify with tubulin and MAPs. In vitro, not all MT are sensitive to mM concentrations of calcium. For instance, the

assembly of MT with tubulin obtained from Ehrlich-ascites tumor cells (74) or Dictiostelium discoideum (75) is not inhibited by the presence of mM calcium. The calcium-resistance of those MT might be a reflection of a particular set of MAPs that may protect MT against calcium.

In vivo, studies have demonstrated that uM amounts of calcium can disassemble MT (22, 76). Since mM amounts of Ca^{2+} are needed to disassemble MT in vitro, in vivo, cells might have a factor that sensitizes cellular MT to uM levels of calcium, and might be the heat labile factor present in crude brain homogenates (73).

In vitro evidence has suggested a possible role of calmodulin in mediating the calcium effects in the disassembly of MT. Marcum et al. (77) showed that calmodulin gave uM calcium sensitivity to assembled brain MT. However, since CaM had to be added in approximately an 80-fold molar excess to microtubular protein, and troponin C was more effective than CaM in the disassembly of MT, it is difficult to accept a role for CaM in the disassembly of MT. High concentrations of S-100 protein also have the ability to promote the disassembly of microtubules (78).

Role of MAPs. Using detergent-permeabilized BSC cells that were supplied with exogenous MAPs, Schliwa et al. (79) showed that CaM antagonists and MAPs prevented the calcium-induced

disassembly of MT. They concluded that MAPs protect MT from calcicalmodulin-mediated disassembly.

Other studies suggest that the calmodulin-dependent disassembly of MT might occur through the interaction of calmodulin with MAP2 and tau. Both MAP2 and tau are calcium-dependent CaMBP, and they bind calmodulin with a low affinity (80). In vitro, MAP2 binds one calmodulin molecule per mol of MAP2. Lee and Wolff (81) and Kotani et al. (82) showed that a high molar excess of CaM to microtubular protein, inhibited the assembly of MT in the presence of calcium. Also, Lee and Wolff (83) demonstrated a deleterious effect of micromolar calcium on the nucleation of MT, which could be reversed by CaM if MAP were omitted from the assay. Sobue et al. (84) suggested the name of "cytocalbins" for those proteins like MAP2, tau and caldesmon, which were able to interact both with CaM and cytoskeletal elements like tubulin or actin. Sobue et al. (84) proposed a 'flip-flop' hypothesis in which calcium-calmodulin interacts with cytocalbins, and thereby eliminates the cytocalbins-cytoskeleton interactions and promotes the disassembly of the latter. At low calcium concentrations, calcium-free CaM should be predominant and cytocalbins would bind to and stabilize the cytoskeleton.

Obviously, this hypothesis must be confirmed in several systems. In this regard Job et al. (85) found that calcium (10^{-6} to 10^{-4} M) causes a concentration dependent-disassembly

of brain MT in vitro in the presence or absence of MAP, but the presence of MAP diminished the calcium effect.

Interestingly, a 20-40 molar excess of CaM to microtubular proteins, either in the presence or absence of MAP, did not enhance the calcium-dependent disassembly of MT. Thus, the "flip-flop" mechanism for the regulation of MT assembly was not confirmed.

Calmodulin may only interact with a subclass of cytoplasmic MT. Job et al. (86) obtained two polypeptides of 56 and 72 kilodaltons from cold-stable MT of rat brain. These proteins (STOP: stable tubule only proteins) could give cold-stability to cold-labile MT. Remarkably, the STOP intrinsic activity was blocked by a calcium-calmodulin dependent phosphorylation of the two proteins. The physiological role of STOP in the dynamics of MT remains to be explored.

Role of protein kinases. Calcium-calmodulin regulates the activity of protein kinase II, which is a major component in the postsynaptic density along with neurofilament protein, fodrin, actin and tubulin (87). Protein kinase II has been identified in several subcellular fractions. The soluble form of the enzyme is able to phosphorylate myosin light chains, synapsin I, tryptophan hydroxylase and, most importantly for the purposes of my thesis, tubulin and MAP2 (88). The in vitro phosphorylation of MAP2 by a

calcicalmodulin-dependent protein kinase impairs the the ability of MAP2 to promote the assembly of MT (88, 89).

Cellular localization of CaM. Immunocytochemical evidence has demonstrated a close association of CaM with the microtubular system of interphase and mitotic cells.

Calmodulin has been localized in basal bodies of several protozoans, like Paramecium and Chlamydomonas, and in epithelial cells of mammals (70, 71). In 1978 Andreasen et al. (47) and Welsh et al. (48) independently reported the intracellular localization of calmodulin in cultured mammalian cells. Calmodulin was found diffusely distributed in the cytoplasm of interphase cells. In prophase, calmodulin was detected in association with the pericentriolar material. At metaphase, calmodulin was localized in the poles of the mitotic spindle and along the MT that end at the kinetochores; tubulin was found in both pole-to-pole fibers and kinetochore fibers. At anaphase, tubulin was still localized in all fibers of the spindle and calmodulin remained at the poles. At late anaphase, calmodulin was found localized also in the MT of the interzonal regions, which condensed during telophase into two well defined regions, one on each side of the cleavage furrow. These observations were subsequently confirmed and extended to several other cell types (90). In a following paper, Welsh et al. (91) reported that cytochalasin B did not

alter the pattern of calmodulin or tubulin in CHO cells. Also, mitotic CHO cells treated with colcemid or N_2O showed an altered distribution of CaM and tubulin. Cold treatment did lead to the disappearance of the MT running from pole-to-pole in mitotic cells, but did not affect those kinetochore MT that had CaM, or the CaM pattern in interphase cells. These observations suggest that CaM was not co-localized with actin filaments, but rather with a stable population of MT during mitosis.

Subsequently, an hypothesis of calcicalmodulin action on microtubules was put forward by Means and Dedman (92) that proposed calcicalmodulin as a possible promoter of disassembly in microtubules in vivo, particularly at the metaphase/anaphase transition in mitotic cells. However, other interpretations are possible. For instance, De Mey et al. (93) questioned the hypothesis because calmodulin is associated with pericentriolar MT at prophase and with pole-to-kinetochore MT during metaphase when there is no visible disassembly of MT.

More recently, immunocytochemical studies have localized calmodulin in dendritic and axonal microtubules (94), and pericentriolar bodies of the centrosome (but not in MT) in interphase 3T3 cells (95). Deery, Brinkley and Means (96) found CaM localized in the colcemid-resistant microtubules and those being reassembled after hypotonic or cold treatments in cultured cells. They concluded that CaM was

co-localized with the stable population of MT during interphase in 3T3 cells. Interestingly, the interaction of CaM with MT remained after an extraction of the cells with up to 10 mM EGTA. Since CaM was associated with MT even in the absence of calcium, Deery et al. proposed that CaM might disassemble MT only during increases in the intracellular calcium concentration. Finally, they observed the disappearance of both CaM association with MT and of the MT themselves upon the exposure of the cells to micromolar concentrations of calcium.

Microinjection. Microinjection experiments have provided contradictory evidence for a possible role of CaM in the disassembly of MT. In one study, neither a two-fold excess of an active fluorescent derivative of CaM nor an antibody of CaM (both injected into living mitotic Ptk² cells) altered the shape of the spindle or the rate of chromosome movement of the recipient cell (97). A second study, on the other hand, showed that the microinjection of calcicalmodulin (but not calcium-free CaM) resulted in local disassembly of cytoplasmic MT in gerbil fibroma cells (98). From this study, however, it is difficult to conclude anything because Ca²⁺ injections of 100 μ M to 1 mM, or calcium-EGTA buffers containing 25 μ M calcium did not disassemble MT. These amounts of calcium injected were high enough to saturate the endogenous calmodulin with calcium, and thus disassembly of

MT should have occurred if CaM were the mediator of MT disassembly.

Calpains

Eukaryotic cells have ~~several~~ proteases that absolutely require calcium for their proteolytic activity. The name Calpains was suggested by Murachi (99) to include all of the calcium-activated proteases such as the calcium-activating factor, kinase-activating factor, calcium-activated neutral proteases and receptor-transforming factor.

Calpains have a cysteine residue in their active site, and neutral pH is required for optimal proteolytic activity. Calpains share partial homology with the papain-thiol-proteases and microbial proteases such as clostripain and streptococcal nuclease (100). It appears that most Calpains are heterodimers with a large subunit of about 80 kD, and a small subunit of about 30 kD (101). The nucleotide sequence of the chicken Calpain has indicated that both subunits have a calcium-binding domain. The catalytic site was found in the large subunit. The calcium-binding domain showed 20-25% homology with the EF hands of the calmodulin family of CaBP. The activity of Calpains can be prevented effectively by endogenous proteins (inhibitors) named Calpastatins and by synthetic inhibitors, such as E-64 and microbial peptides like leupeptin (99).

Cytoskeletal components are known to be substrates for Calpains in vitro (102). Calpains have among their substrates the cytoskeletal proteins like troponin, alpha-actinin, vimentin and MAP2. In 1975, Sandoval and Weber (18) described the calcium-dependent cleavage of MAP2 by an enzyme obtained from bovine brain. They also showed that the calcium-dependent proteolysis of MAP2 resulted in the irreversible disassembly of MT. The enzymatic activity was found mainly in the soluble fraction of a bovine brain homogenate, and did not co-purify with MT during cycles of assembly and disassembly. Klein et al. (103) further characterized this enzymatic activity. They partially purified the enzyme and found that 2 μ M calcium chloride was required for half-maximal activation. The same authors also showed that the enzyme required a pH of 6.5 to 7.5 for optimal activity, and that the enzymatic activity was heat-labile.

Thus, the calcium-dependent proteolysis of MAP2 and the resulting disassembly of MT are well documented in in vitro systems. However, there is still no available evidence that Calpains mediate the calcium-induced disassembly of MT in vivo.

Lymphocytes

Lymphocytes are the effector cells of the immune system. There are two types of lymphocytes, B and T, and both are derived from progenitor cells in the bone marrow (for general information on lymphocytes see 104). B cells mature in the bone marrow before they migrate to the lymphatic organs and spleen, and are responsible for the production of antibodies. B cells have a few of their antibodies on the outside face of the plasma membrane (Ig^+). In mammals T cells mature in the thymus and migrate through the entire lymphatic tissue, spleen and blood. T cells do not have immunoglobulins on their surface (Ig^-). The main function of T cells appears to be the modulation of the activity and proliferation of B cells.

Mitogenic stimulation of T lymphocytes.

In vivo, T cells are induced to proliferate when they become activated by the binding of their respective antigen. The activated T lymphocyte undergoes metabolic and morphologic changes named blastogenesis. The resulting cell is called blast, which ultimately divides, amplifying the immune response. Thus, the proper response of T cells by foreign molecules or antigens, results in an increased cell proliferation of both T and B cells, production of secretion

of antibodies, and finally degradation and elimination of the foreign molecule.

In vitro, T lymphocytes also can be induced to proliferate by plant lectins such as concanavalin A (con A) and phytohemagglutinin. The in vitro activation of lymphocytes is now widely used as a model system to study diverse aspects of lymphocyte function. Lectin activation of T lymphocytes in culture results in changes in membrane permeability (105), increases in phosphatidylinositol turnover (105), intracellular calcium concentration (107), cyclic nucleotide levels (108), RNA and protein synthesis (109), ADP ribosylation (110) and DNA repair (111), transcription of cellular oncogenes (112, 113), increase in cell size, enlargement of nucleus and disaggregation of chromatin (114, 115) and DNA synthesis and mitosis (116).

The in vitro response to mitogens by lymphocytes is asynchronous; therefore, any biochemical analysis of the activated population of lymphocytes may represent, at best, a misleading average of the different events occurring in each different subpopulation of lymphocytes. Setterfield et al. (114) stressed the need to correlate when possible cytological studies with the biochemical events in blastogenesis. They measured the extent of the lymphocyte stimulation according to nuclear and cellular morphologies. Most of the resting lymphocytes, designated morphotype I, were approximately 6 μ m in diameter, and had small nuclei,

nucleoli and large aggregates of chromatin. After 48h of the mitogenic stimulation by concanavalin A, a large proportion of lymphocytes was fully stimulated, or morphotype III, measured approximately 10 to 12 μ M in diameter, and had large nuclei, large nucleoli and disaggregated chromatin in small clumps. Partially stimulated lymphocytes, or morphotype II, had intermediate characteristics between morphotypes I and III.

The microtubular system of lymphocytes.

Resting lymphocytes have a sparse microtubular system (4). Electron micrographs shown by Rudd et al. (117) indicated that concanavalin A-stimulated cells had an increased number of MT. Waterhouse, Anderson and Brown (118) did direct counts of MT initiated from the centrosome of stimulated mouse T lymphocytes. They showed a five-fold increase in the number of MT in fully stimulated cells, as compared to resting cells. Waterhouse et al. (118) proposed that the increased number of MT in stimulated lymphocytes was dependent on the 50% increase in the tubulin content of those cells. Schweitzer and Brown (119) examined the changes in the organization and MT assembly capacity of the centrosome during the mitogenic stimulation of mouse T lymphocytes. Stimulated lymphocytes showed a reorganization of the centrosome, an increased number of satellite bodies and an

increased capacity to initiate MT from the centrosome. Schweitzer and Brown (119) used cold or colchicine treatments to disassemble MT in intact cells. Cold treatment resulted in the disassembly of MT and disaggregation of the satellite bodies. If reassembly of MT was allowed at 37°C, there was a reaggregation of satellite bodies. Colchicine treatment did not cause any alteration in the number or organization of satellite bodies, despite the absence of MT.

An intact microtubular system of lymphocytes has been implicated in several functions in these cells, including mitogenic responses (120), redistribution of cell surface molecules (121, 122) and intercellular contacts (123). It has also been proposed that not only the presence of an intact microtubular system, but also the organization in the cytoplasm might be essential for some functions of the lymphocyte (4).

Thus, here it should be clear that the mitogenic stimulation of lymphocytes induces the microtubular system to exhibit remarkable changes. The changes on the microtubular system can be further manipulated by the use of several drugs and changes in temperature. For these reasons, lymphocytes provide a suitable model for the examination of the role of putative regulatory molecules (i.e. calcium, calmodulin) on the organization of the microtubular system. This model system also provides an opportunity to examine how changes in the organization of the microtubular system affect some

functions of the lymphocyte (i.e. cytotoxicity, cell-to-cell recognition and proliferation).

Calcium and calmodulin in the lymphocyte.

During the past few years, it has become evident that calcium and calmodulin might be key elements in diverse functions of the lymphocyte. The interest in the role of calcium in the lymphocyte began with the demonstration of an extracellular calcium requirement for the proliferation of rat thymocytes in vivo and in vitro (124, 125). Later, Whitney and Sutherland (126) suggested that a calcium influx occurring early after the lectin activation of lymphocytes, was required for those cells to proliferate. Recently Tsien et al. (127) and Hesketh et al. (128) measured the intracellular free calcium concentration in lymphocytes. They found that resting lymphocytes had a free calcium concentration of 120 nM, and the mitogenic stimulation of lymphocytes resulted in a transient increase in the intracellular free calcium concentration to 240 nM. This increase in the intracellular calcium concentration was found to be totally dependent on the extracellular calcium concentration. An increase in calcium influx was detected by Wolff et al. (129) in lymphocytes after mitogenic stimulation by lectins. The increase in the calcium influx peaked at about 24h after the stimulation. It has been suggested that

the increased intracellular calcium content might be required for the production of interleukins (130).

Lymphocytes have several CaBP in addition to CaM (131). It is estimated that CaM constitutes approximately 0.58% of the total protein in lymphocytes (132). The amount of CaM per cell doubles after 24 hours of lectin activation (132, 133). The increase of CaM content in lymphocytes is apparently due to a post-transcriptional event since the total amount of translatable CaM mRNA does not appear to change upon activation (134).

Studies with CaM antagonists suggest that lymphocyte-mediated cytolysis is CaM-dependent (135). Calmodulin might also be involved in the activation process of T lymphocytes. Trifluoperazine or W-7, added within the first hour after the activation, blocked ^3H -thymidine uptake and blastogenic transformation (132, 133, 136, 137). Furthermore, cyclosporin A, a potent immunosuppressive drug that inhibits the activation process of T lymphocytes, has been found to bind CaM in vitro (138). Trifluoperazine and calmidazolium inhibited the binding of cyclosporin A to CaM. These results suggest that a CaM-dependent event is required for the proper activation and proliferation of T lymphocytes.

Surprisingly, the localization of CaM in lymphoid cells has remained largely unexplored. Calmodulin was found in submembranous patches after the induction of capping in mouse T lymphoma cells (139). Bourguignon et al. (140) proposed

proposed that CaM might modulate the activity of myosin light chain kinase (MLCK). This enzyme phosphorylates the light chains of myosin, and this might be a requirement for the formation of the caps in lymphoid cells. Salisbury et al. (141) proposed that CaM also might be involved in the formation of clathrin-coated pits, since TFP blocks this process in lymphoid cells. Therefore, CaM activity appears to be involved in several functions of the lymphocyte at the membrane level.

Bachvaroff et al. (132) used immunofluorescence to detect CaM in porcine lymphocytes. They found CaM diffusely distributed in resting, stimulated and mitotic lymphocytes. Although the authors claimed that CaM was localized mainly in the Golgi region in stimulated lymphocytes, there was no visible indication of that claim. Whether or not there is any particular localization or redistribution of CaM in lymphocytes during blastogenesis and mitosis, therefore, remains an open question.

Hypothesis

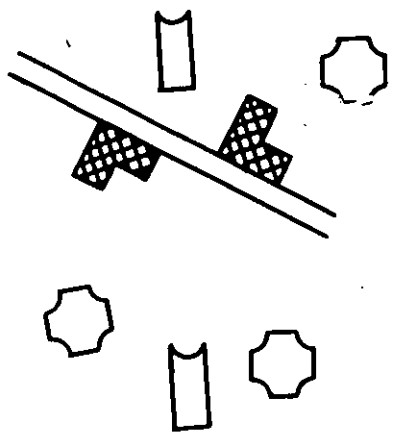
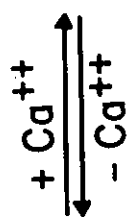
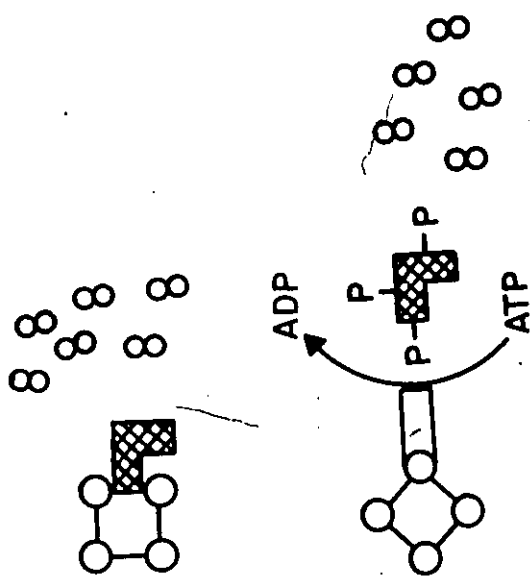
The aim of my research was to investigate the subcellular localization of CaM and its relationship with the dynamic changes of the microtubular system during the mitogenic stimulation of mouse T lymphocytes.

Figure 2 illustrates the current view on how calcicmodulin interacts with microtubular components such as MAPs to promote the disassembly of MT in vitro. It has been proposed that calcicmodulin might interact with some MAPs and, in this way, shift those proteins away from the MT. Alternatively, calcicmodulin might affect the stabilizing capacity of MAPs or STOP by promoting their phosphorylation by calcicmodulin-dependent kinases. In both cases, the net effect is the disassembly of MT. The view of CaM as a negative regulator of the dynamics of MT, has also been largely supported by the well known localization of CaM in some MT of mitotic cells.

In this thesis I propose that if CaM is a regulatory molecule of the microtubular system, the localization of this protein should closely correlate with the dynamic changes of the microtubular system that occur during the mitogenic stimulation of mouse T lymphocytes. Single and double immunofluorescence staining with monospecific antibodies to CaM and tubulin, were used to study the temporal relationships between CaM and MT during the stimulation and mitosis of T lymphocytes. Since the distributions of both CaM and MT in 3T3 cells have been well characterized, these cells were used occasionally to corroborate and to expand some of the observations obtained with lymphocytes. Disturbances of the microtubular system of lymphocytes by CaCl_2 , EGTA, colchicine, colcemid, taxol, and CaM-antagonist

Figure 2. Current view of the different models of action of Ca^{2+} - CaM on MT.

7



// : Microtubule
 ○ : Calcium
 8 : Tubulin dimers

[] : Protein kinase
 [] : Calmodulin
 [] : MAP

CaM-antagonist additions, and temperature changes were used to examine the local and temporal relationships between CaM and MT. Limited cell permeabilization was used to artificially modulate the intracellular free-calcium concentration in order to study the stability of the different types of MT to calcium, CaM antagonists, and protease inhibitors.

Finally, based on the results obtained during this research, I propose an alternative view of the mode of action of calcium, calmodulin, MAPs and Calpains in the dynamics of MT.

MATERIALS AND METHODS

Reagents

The chemicals used were reagent grade. The water used was distilled and deionized. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal calf serum were obtained from Flow Laboratories, Inc. (Mississauga, Ont.). Concanavalin A was obtained from Calbiochem (San Diego, CA). 1, 4-Piperazine-N, N'-bis-ethane sulfonic acid (PIPES), ethylene glycol-bis-(beta-aminoethylether) N,N,N', N'-tetraacetic acid (EGTA), Triton X-100, polyoxethylene 20 cetyether (Brij-58), bovine serum albumin (BSA), p-phenylenediamine, GTP, phenylmethyl-sulfonyl-fluoride (PMSF), leupeptin, soybean trypsin inhibitor, trifluoperazine (TFP), N-(6-aminohexyl)-1-naphtalenesulfonamide (W-5), N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W-7), colchicine and colcemid were obtained from Sigma Chemical Co. (St. Louis, MO). Hoechst 33258 was obtained from American Hoechst Corp. (San Diego, CA). Calmidazolium (CDZ) was obtained from Janseen Life Sciences Products (Beerse, Belgium). Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Paraformaldehyde and glutaraldehyde were purchased from J.B.S. Chemical Inc. (Dorval, Qué). Acrylamide, N,N'-methylene-bisacrylamide, trizma base (Tris: tris hydroxymethyl aminomethane) glycine,

nitrocellulose (NTC; pore 0.45 μ m), low-molecular weight markers, 4-chloro-1-naphtol, AFFIGEL phenothiazine, Bradford Reagent (protein determination) and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Missisagua, Ont.). Gelatin, CaCl_2 (anhydrous, 98% purity), polyethyleneglycol (PEG) and polyoxyethylene-sorbitan monolaurate (Tween 20) were purchased from J.T. Baker (Phillipsburg, NJ).

Antibodies

Affinity-purified sheep anti-CaM IgG was obtained from Biomedical Technologies Inc. (Cambridge, MA). Mouse monoclonal antibody to tubulin (ascites fluid) 1D9, (IgG) was kindly provided by William A. Aitchison. Rabbit anti-mouse Ig, and FITC (Fluoresceinisothiocyanate) conjugated rabbit anti-sheep IgG were obtained from Cedarlane Labs (Hornby, Ont.). TRITC (tetramethylrhodamine-isothiocyanate) conjugated rabbit anti-mouse IgG. FITC-labelled goat anti-mouse IgG, horse radish peroxidase (HRP)-conjugated rabbit anti-sheep IgG were obtained from Cappel-Cooper Biomedical (West Chester, PA). Biotinylated horse anti-mouse IgG was obtained from Vector Laboratories Inc. (Burlingame, CA). Streptavidin-biotinylated horse radish peroxidase complex was obtained from Amersham (Arlington Heights, IL).

Cell culture

Mouse splenocytes were isolated from male Balb/c mice 8 to 12 weeks old, as described previously (118). Spleens were aseptically removed from the mice and disrupted on a wire screen into 4 ml of medium (RPMI 1640 media containing 2 mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin), centrifuged at 1500 r.p.m. in a clinical centrifuge. Supernatant was discarded and cells resuspended in 5 ml of 0.17M NH_4Cl for 10 min to lyse red blood cells. Then, cells were resuspended in 10 ml of calf serum, centrifuged at 1500 r.p.m. and the supernatant containing red cell membranes was discarded.

T cell enrichment was done according to Mage et al. (142). Isolated lymphocytes were resuspended in 15 ml of phosphate-buffered saline (PBS) plus 5% fetal calf serum at approximately 4×10^6 cells/ml and plated on a 100 mm^2 petri dish previously coated with rabbit anti-mouse Ig (1:1000 in 50 mM Tris) for one hour and washed thoroughly with PBS. The mixed population of lymphocytes was incubated in the coated petri dish for one hour at 37°C ; non-attached cells were decanted and centrifuged at 1500 r.p.m., supernatant discarded and the enriched T cell pellet resuspended in the appropriate volume of complete medium for their culture.

To determine the purity of the cell suspension, 200 ul of the cells were put on a half coverslip previously coated

with 0.1% poly-L-lysine (see Immunofluorescence); the coverslip with the attached cells was then washed in PBS, fixed for 20 min with 3% paraformaldehyde-PBS, rinsed in PBS (3 X 4 min) and incubated for one hour with a fluorescein-labelled goat anti-mouse IgG (H + L) at 1:100 dilution. The cells were washed in PBS (3 X 4 min) and mounted on slides containing 8 ul of mounting media (0.1% of phenylenediamine, 50% glycerol in PBS, pH 7.8). Each slide was then observed with a microscope equipped for epifluorescence (see Microscopy). At least 200 cells were examined to determine the percentage of Ig⁺ (B) cells. Routinely, this method of T cell enrichment results in approximately 18% contamination of B cells, and this level drops to less than 5% after 24h of culture.

The T cell-enriched suspension was cultured for various lengths of time in sealed culture flasks at 37°C, at a density of 2×10^6 cells/ml in RPMI 1640 buffered to pH 6.9 with 20 mM Hepes, and supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin. T cells were then stimulated by the addition of 4 ug/ml of con A. Cultures were supplemented every 24h with a 10% volume of complete medium to improve cell viability.

Mouse 3T3 cells were cultured on half coverslips at 37°C and 5% CO₂ in DMEM with 10% FCS. For immunofluorescence, cells were plated at a density of about 0.5×10^6 cells/ml

and coverslips used at 24h, before the cultures reached confluency.

Immunofluorescence

All procedures were at room temperature except when indicated. T lymphocytes were attached to half coverslips previously coated with poly-L-lysine. Half coverslips were flooded with 0.1% poly-L-lysine for approximately 10 min and then rinsed with water and air dried. Two hundred μ l of cells were then put on the coverslip and left to settle for about 10 min before the immunostaining. In all of the protocols, the appropriate dilutions of antibodies were determined empirically. The appropriate controls were used to determine that the fluorescence was due to the complex of the first antibody with the labelled second antibody, and not due to autofluorescence of cells or to non-specific binding of the labelled second antibody.

Protocol I.

Coverslips with the attached cells were briefly (30 sec.) washed in PBS and fixed in 3% paraformaldehyde for 20 min. Cells were then permeabilized in 0.2% Triton X-100-PBS for 20 min. Coverslips with the attached cells were washed three times (3 X 4 min) in sodium borohydride (1 mg/ml) to

reduce the residual free aldehydes. Cells were then washed three times (3 X 4 min) in PBS and inverted on 20 ul of sheep anti-CaM IgG at 20 ug/ml. After one hour, cells were rinsed in PBS (3 X 4 min) and flooded with 100 ul of FITC-labelled rabbit anti-sheep IgG at 1:100 dilution in PBS. The cells with the FITC-labelled antibody were incubated for one hour, and then washed in PBS (3 X 4 min). All samples were stained for DNA by a 2 min incubation in 1.5 ug/ml Hoechst 33258 in PBS (143). The coverslips were then mounted on slides with 8 ul of mounting medium and sealed with nail polish.

Protocol II.

Cells attached to coverslips were washed in fresh Hank's balanced salt solution (HBSS: 1.2 mM CaCl_2 , 6.0 mM glucose, 137 mM NaCl, 0.8 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, 0.4 mM KH_2PO_4 , 0.3 mM Na_2HPO_4 , 3.2 mM NaHCO_3 , 6.0 mM KCL, 0.8 mM $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$) for 30 sec. Coverslips were then washed for 30 sec. in cytoskeletal buffer (C.B.: HBSS supplemented with 2 mM $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$, 2 mM EGTA, 5 mM PIPES) pH 6.9 plus 4% polyethylene-glycol (PEG). The cells were then permeabilized for 90 sec. in 0.05% Triton X-100 + 4% PEG in C.B. and washed for 30 sec. in C.B. containing 4% PEG. The cells on coverslips were then fixed for 20 min in 3% paraformaldehyde + 0.25% glutaraldehyde + 4% PEG in C.B., reduced in 1 mg/ml sodium borohydride in PBS (3 X 4 min), and washed in PBS (3 X 4

min). The coverslips were then incubated with sheep anti-CaM (as described above) for one hour. For single immunofluorescence staining, cells were then processed as described in protocol I. For double immunofluorescence staining, cells were incubated for 20 min in 0.15% gelatin-PBS, after the incubation and wash of the first antibody (i.e. sheep anti-CaM), and then washed in PBS (3 X 4 min). Subsequently, coverslips were incubated with the appropriate first secondary antibody (i.e. FITC-labelled rabbit anti-sheep IgG 1:100). Next, cells were washed in PBS (3 X 4 min), incubated in 0.15% gelatin-PBS for 20 min and washed in PBS (3 X 4 min). Cells were then incubated for 1 hour with the second primary antibody, 100 ul of anti-tubulin in a 1:25 dilution in PBS. Cells were washed in PBS (3 X 4 min) and then incubated with the second labelled antibody rhodamine-labelled goat anti-mouse IgG (adsorbed in mouse liver acetone powder and diluted 1:5 in PBS) for 45 min. Cells were washed in PBS (3 X 4 min), stained for DNA and mounted on slides as described for protocol I.

Protocol III.

During the last ten minutes of the different treatments described in the section on Results (The effects of CaM antagonists on microtubules), 200 ul of cells were attached to poly-L-lysine-coated half coverslips. Attached cells were

put directly into 3% paraformaldehyde-0.25% glutaraldehyde in C.B. for 20 min. Cells were briefly washed in C.B. and then permeabilized for 2 min in 0.2 Triton X-100-C.B. After the permeabilization step, coverslips were processed identically as in protocol I, except that the primary antibody used was the anti-tubulin (1:25 in PBS), and the second labelled antibody used was FITC-labelled goat anti-mouse IgG (diluted 1:100 in PBS).

Protocol IV.

In this protocol, cells attached to coverslips were briefly washed in PBS and then washed for 30 sec. in C.B. Cells were then washed for 2 min in the appropriate calcium-EGTA buffer (see Ca-EGTA buffers). Next, cells were permeabilized for 3 min in 0.15% Brij-58 in the calcium EGTA buffers. Cells were then equilibrated for 10 min with the desired calcium-EGTA buffer. Cells were either directly fixed in 3% paraformaldehyde-0.25% glutaraldehyde in the same calcium-EGTA buffer, or alternatively, cells were first washed in an EGTA buffer containing less than 10^{-8} M calcium, transferred for 15 min in a reassembly buffer (R.B.: 40 mM PIPES, 1 mM Mg^{2+} , 1 μ M Ca^{2+} , 1 mM Mg-GTP) at pH 6.9, and then fixed in a calcium EGTA buffer (1 μ M Ca^{2+}) plus fixatives as described above. Cells were further permeabilized for 2 min in 0.5% Triton

X-100 in PBS and processed according to protocol I for anti-tubulin immunofluorescence.

Microscopy.

Cells were examined with a Zeiss Universal microscope equipped for epifluorescence illumination and phase microscopy. All observations were with an oil 100 X neofluor objective (1.3 numerical aperture), and recorded with XPl-400 (Ilford) film.

Calcium EGTA buffers

Calcium-EGTA buffers were prepared in 40 mM PIPES and 10 mM EGTA according to formulae from Steinhardt et al. (144) and Gilson et al. (145), assuming an association constant for EGTA and calcium of $10^{10.7}$ at pH 6.9. Calcium additions were done from a 0.1 M stock solution of CaCl_2 calibrated with a calcium-sensitive electrode (Orion). Magnesium was added as MgSO_4 to give a final concentration of 1 mM Mg^{2+} . < 0.01 μM Ca^{2+} : 1.33 mM MgSO_4 , no CaCl_2 ; 0.1 μM Ca^{2+} : 1.28 mM MgSO_4 , 1.51 mM CaCl_2 ; 1.0 μM Ca^{2+} : 1.12 mM MgSO_4 , 6.40 mM CaCl_2 ; 10 μM Ca^{2+} : 1.02 mM MgSO_4 , 9.48 mM CaCl_2 ; 50 μM Ca^{2+} : 2.2 mM MgSO_4 , 11.53 mM CaCl_2 . 0.10 mM Ca^{2+} and 1.0 mM Ca^{2+} concentrations were achieved by direct addition of CaCl_2 from the stock solution in the same 40 mM PIPES but with no EGTA present;

MgSO₄ was kept at 1 mM. pH was carefully adjusted to 6.94 ± 0.02 with NaOH. CaCl₂ and MgSO₄ stock solutions were kept in plastic bottles, and all solutions were prepared fresh before each experiment.

Calmodulin purification

Calmodulin was purified from freshly isolated bovine brain according to Jamieson and Vanaman (147) and MacManus (148). The procedure involved a 50% ammonium sulfate precipitation at pH 4.0, followed by affinity chromatography in Affigel-phenothiazine and anionic exchange in DEAE-Sephadex A-25. The calmodulin obtained was homogeneous by 10% SDS-PAGE.

Immunoblotting

Proteins from cell or tissue extracts were separated in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the system described by Laemmli (146), and then transferred to nitrocellulose (NTC) for immunoblotting according to Van Eldik and Wolchok (149).

Stimulated mouse T lymphocytes cultured for 48h, heart, kidney and intestine (all from mice) were prepared as follows. All samples were washed with cold PBS and then quickly homogenized in 1 ml of PBS containing 0.50 mM PMSF at

4°C, with a teflon-glass homogenizer. A small aliquot of each sample was kept at -20°C for protein determination. Homogenates were then diluted with 1 ml of 2X electrophoresis sample buffer (62.5 mM Tris, 10% glycerol, 2% SDS, 10 mM EGTA, 5% beta-mercaptoethanol, 0.005% bromophenol blue) and immersed in boiling water for 3 minutes. Forty micrograms of protein were loaded per lane on the SDS gel.

3T3 cells growing in 100 mm² dishes were experimentally manipulated as described in the Results section (The effect of 0.1 mM calcium on cellular tubulin). 500 µl of electrophoresis sample buffer were added, and cells were scrapped with a rubber policeman, homogenized and immersed in boiling water for 3 minutes. Twenty-five microliters of each homogenate were loaded per lane on the SDS gel.

The SDS-PAGE was run on 0.5 mm thick slab gels. The separating gel (approx. 12 cm long), 10% acrylamide in 0.375M Tris and 0.1 SDS, pH 8.8, was polymerized with TEMED and ammonium persulfate. The stacking gel was 4% acrylamide (approx. 2 cm long), 0.125M Tris, 0.1% SDS, pH 6.8 and polymerized with TEMED and ammonium persulfate. The electrophoresis was run at 120V with a buffer of 0.025M Tris, 0.19M glycine, 0.1% SDS at pH 8.3 until the bromophenol blue reached the bottom of the gel. Gels with the separated proteins were then laid on top of a sheet of NTC and put into a transfer chamber (Bio-Rad) for 1h at 180 mA. The transfer buffer used was 0.025M Tris, 0.19M glycine, 20% methanol,

1.0 mM CaCl_2 . A piece of NTC was stained for 5 min with 0.1% (w/v) amido black in 45% (v/v) methanol, 10% (v/v) acetic acid, and destained with running water.

Unstained NTC was immediately dried for 10 min at 80°C and then fixed for 45 min in 0.25% glutaraldehyde-PBS. NTC was blocked for 2 hours with 2% bovine serum albumin (w/v)-0.2% gelatin (w/v) in PBS, at 37°C , and then incubated for 2 hours in either sheep anti-CaM IgG (10 - 20 $\mu\text{g}/\text{ml}$ of 0.1% gelatin-PBS pH 7.0) or 1D9 (1:50 dilution in 0.1% gelatin-PBS pH 7.0) at 37°C . NTC was washed two times (10 min each time) with 0.05% Tween-20-PBS and three times (10 min each time) with PBS. The NTC blotted with sheep anti-CaM was incubated for 1h with horse radish-peroxidase conjugated rabbit anti-sheep IgG, diluted 1:1000 in 0.15% gelatin PBS and then washed with Tween-20-PBS and PBS as described above. The NTC blotted with 1D9 was incubated for 1h with a biotinylated horse anti-mouse IgG, diluted 1:500 in 0.15% gelatin PBS and then washed as described above. Subsequently, the 1D9 blotted NTC was incubated for 30 min with a streptavidin-biotinylated horse radish peroxidase, diluted 1:400 in 0.15% gelatin-PBS and washed as before. Anti CaM and 1D9 binding to NTC were detected with 3 mg/ml of 4-chloro-1-naphtol in PBS containing 0.006% hydrogen peroxide. The reaction was stopped after 30 min by washing with water.

Miscellaneous

Cell viability was determined by the trypan blue exclusion test (150): Protein determinations were done with the dye-binding assay (Bio-Rad; 151).

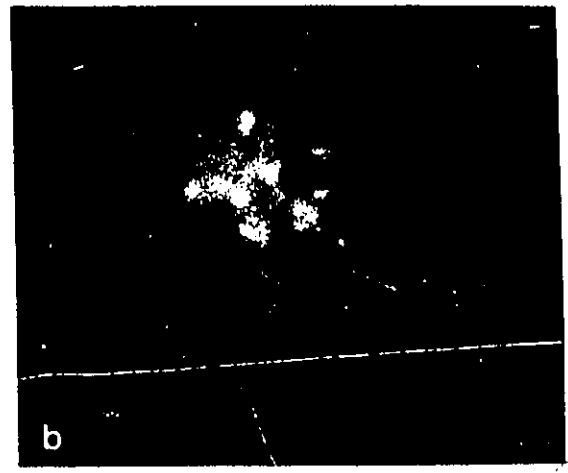
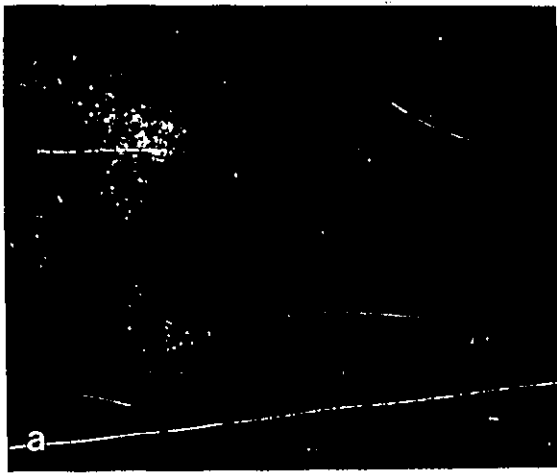
RESULTS

Characterization of anti-calmodulin and anti-tubulin
antibodies

The polyclonal antibody against CaM was an IgG raised in sheep. According to the supplier, this antibody was affinity purified and failed to produce a precipitation line by Ouchterlony. Since the pattern of CaM in 3T3 cells has been extensively studied, these cells provide an excellent first positive control. The first step was to confirm if the anti-CaM could detect CaM in the pattern already known for 3T3 cells. 3T3 cells were fixed in paraformaldehyde and then permeabilized with Triton X-100 (protocol I). Interphase 3T3 cells showed a diffuse distribution of CaM in cytoplasm with no apparent staining within the nucleus (Fig. 3a, b). 3T3 cells during metaphase and anaphase had CaM associated with the spindle apparatus (Fig. 3c, d). Liquid phase adsorption of the anti-CaM with a 60-fold molar excess of electrophoretically pure bovine brain CaM resulted in no immunofluorescence detection of CaM in 3T3 cells (Fig. 3f). Next, I used the anti-CaM and protocol I (fixation of paraformaldehyde and permeabilization in Triton X-100) to study the CaM localization in mouse T lymphocytes. Con A-stimulated mouse T lymphocytes at 48h, in interphase, showed a diffuse immunofluorescent staining of CaM in their

Figure 3. Immunofluorescence staining of CaM in 3T3 cells.

Cells were processed as indicated in protocol I and stained for CaM. a and b are an interphase cell; c, d, e and f are mitotic cells. a and c are CaM staining with anti-CaM; e CaM staining with antibody adsorbed for 24h at 4°C with a 60-fold molar excess of bovine brain CaM; and b, d and f are DNA staining. Bar = 1.0 um.



cytoplasm (Fig. 4a, b). Lymphocytes during prophase showed CaM localized as two circles at both sides of the chromosomal mass in addition to a diffuse CaM staining (Fig. 4c, d). The liquid adsorption of the anti-CaM with authentic bovine brain CaM, abolished the staining of CaM in the lymphocytes at 48h after the stimulation with con A (Fig. 4e, f).

The antibody to tubulin was a monoclonal IgG supplied as ascites fluid (W.A. Aitchison, personal communication). This antibody recognized the microtubular array by immunofluorescence in both 3T3 cells and mouse T lymphocytes.

The specificity of both anti-CaM and anti-tubulin antibodies was done by immunoblotting. Several tissues and 48h stimulated T lymphocytes were obtained from Balb/c mice as described in Materials and Methods. Because of the acidic nature and low molecular weight of CaM, the immunoblotting presented some unusual difficulties. Essentially, the immunoblotting procedure was done according to Van Eldik and Wolchok (149), except that 1.0 mM CaCl_2 was added to the transfer buffer, and the nitrocellulose with the adsorbed proteins dried at 60°C for 15 min immediately after the transfer to increase the efficiency of CaM retention on nitrocellulose. As shown in figure 5, this particular immunoblotting procedure gives good transfer of both high and low Mr proteins of all tissue homogenates, including CaM (Fig. 5, 1 to 5). The anti-CaM detected a single component of total cell homogenates of lymphocytes, heart, kidney and

Figure 4. Immunofluorescence staining of CaM in lymphocytes.

Cells were processed as indicated in protocol I and stained for CaM. a, b, e and f are a 48h stimulated lymphocyte at interphase; c and d are a mitotic lymphocyte. a and c are CaM staining with anti-CaM; e CaM staining with antibody adsorbed for 24h at 4°C with a 60-fold molar excess of bovine brain CaM; and b, d and f DNA staining. Bar = 1 um.

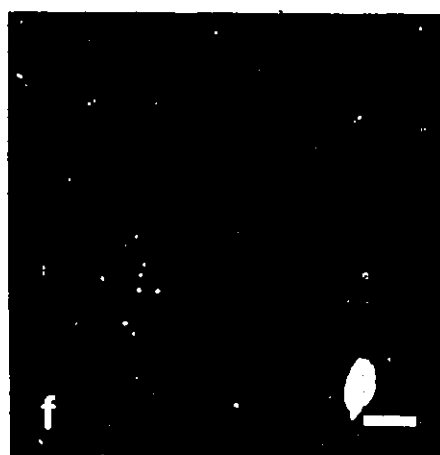
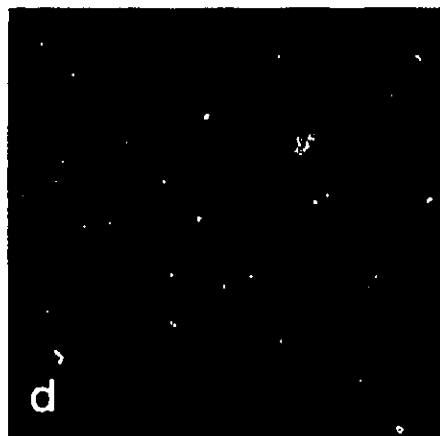
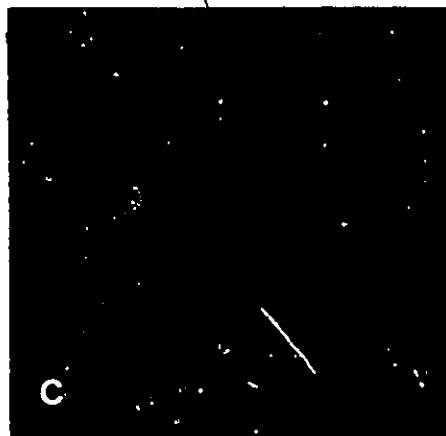
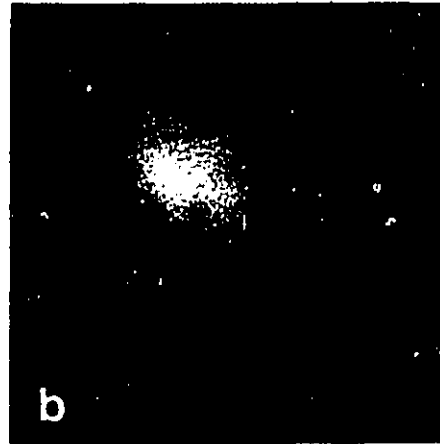
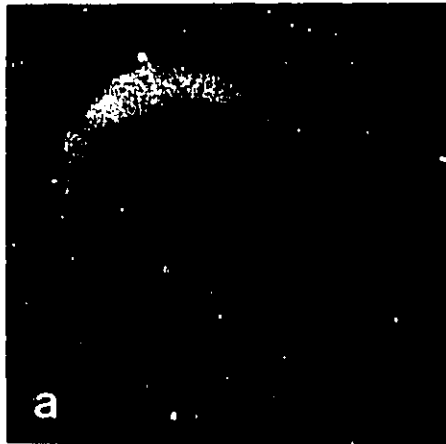
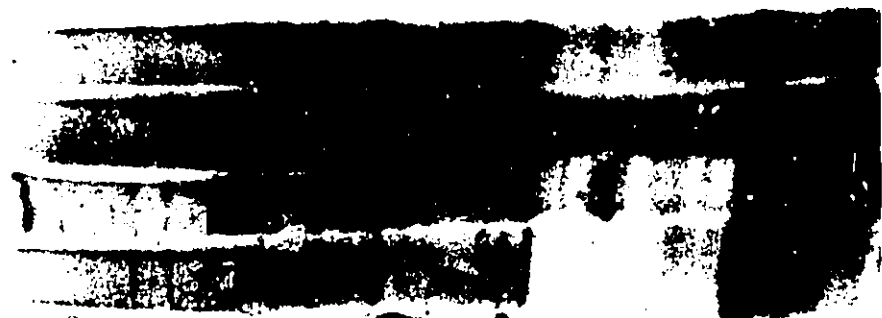


Figure 5. Immunoblotting of anti-CaM and anti-tubulin antibodies against different tissues from Balb/c mouse.

Lane S to 5 total proteins transferred to NTC stained with amido black. S, molecular mass markers from top to bottom are: 92 kD, 66 kD, 45 kD, 31 kD, 21.5 kD and 14.4 kD. Lanes 1' to 5', antigens detected with sheep anti-CaM, Lanes 1" to 3" antigens detected with mouse anti-tubulin. Lanes 1, 1' and 1", 48h stimulated lymphocytes. Lanes 2, 2' and 2", heart. Lanes 3, 3' and 3", kidney. Lanes 4 and 4', intestine. Lanes 5 and 5' bovine brain calmodulin. Anti-CaM and anti-tubulin were detected with second antibodies labelled with horse radish peroxidase and 4-chloro-1-naphtol as substrate.

S 1 2 3 4 5 1' 2' 3' 4' 5' 1" 2" 3"



C

bovine brain CaM with Mr 17.5 kD (Fig. 5, 1' to 5'). Anti-tubulin 1D9 recognized a single component of Mr 52.6 kD in total cell homogenates of lymphocytes and kidney (Fig. 5, 1", 3"). The reason for the lack of immunoreactivity of 1D9 with heart homogenates (Fig. 5, 2") is not known.

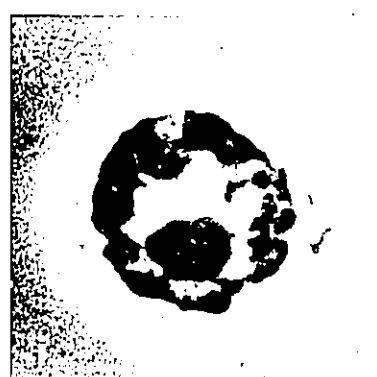
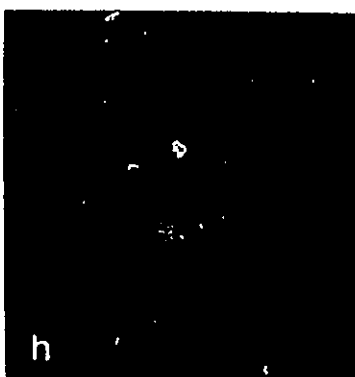
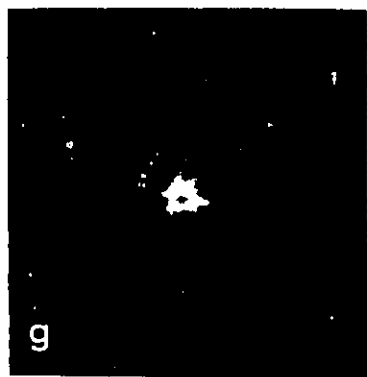
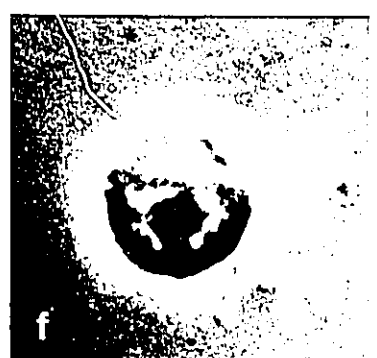
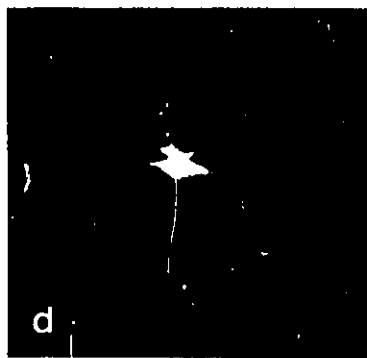
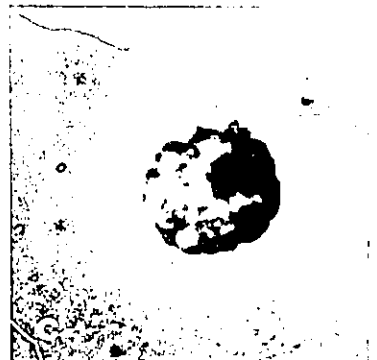
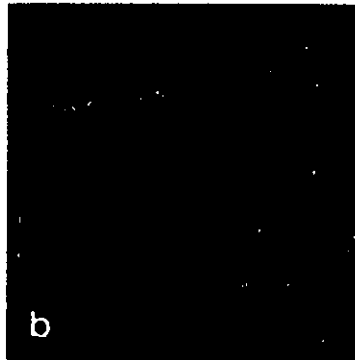
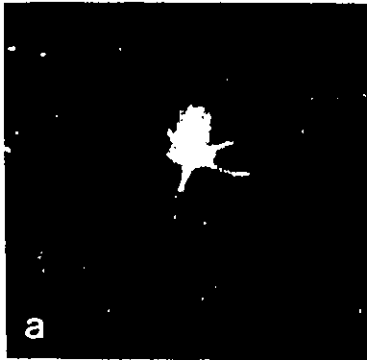
The stimulation of lymphocytes by concanavalin A

Balb/c splenocytes were obtained and enriched for T cells as described in Materials and Methods. Since mouse T cells do not have Ig molecules on their surfaces, Ig⁺ cells (B cells) were easily adsorbed on a petri dish coated with rabbit anti-mouse Ig. The Ig⁺ cells remaining after the adsorption were 18% but declined to only 8% after 24 hours to less than 5% after 48h in culture.

Indirect immunofluorescence with the anti-tubulin 1D9 was used to detect the changes in the microtubular system of mouse T lymphocytes at different times after the stimulation with con A. Before staining with the antibody, cells were briefly permeabilized in Triton X-100 and then fixed with aldehydes (protocol II, see Materials and Methods). This procedure is a modification of the one described by Deery et al. (96) which has been successful in demonstrating the localization of CaM in the MT system of interphase 3T3 cells. Resting lymphocytes showed a very sparse microtubular system with only a few MT radiating from a common site (Fig. 6a, b,

Figure 6. Immunofluorescence staining of tubulin in resting, 24h and 48h stimulated lymphocytes.

a, b, and c, resting lymphocyte. d, e and f, 24h stimulated lymphocyte. g, h and i, 48h stimulated lymphocyte. a, d and g tubulin staining. b, e and h, DNA staining. c, f, and i, phase microscopy. Resting and con A stimulated lymphocytes were processed according to protocol II. Anti-tubulin was detected with FITC-goat anti-mouse IgG. Bar = 1.0 um.



c). Partially stimulated lymphocytes after 24h of con A addition (Fig. 6d, e, f) did not show a major change in their microtubular system if compared to resting lymphocytes (Fig. 6d vs. 6a). Partially stimulated lymphocytes still had a sparse microtubular system. However, fully stimulated mouse T lymphocytes at 48h after the addition of con A exhibited an increased microtubular system, and all MT were radiated from a common location presumed to be the centrosome (Fig. 6g, h). The increased MT system of 48h stimulated T lymphocytes correlated with their large nucleolus, dispersed chromatin and increased cell size observed by phase microscopy (Fig. 6i). Since T cell blastogenesis induced by concanavalin A was an asynchronous response, phase microscopy was always useful to determine whether a particular distribution of tubulin or CaM corresponded to a fully stimulated cell.

The distribution of CaM and MT during stimulation of lymphocytes

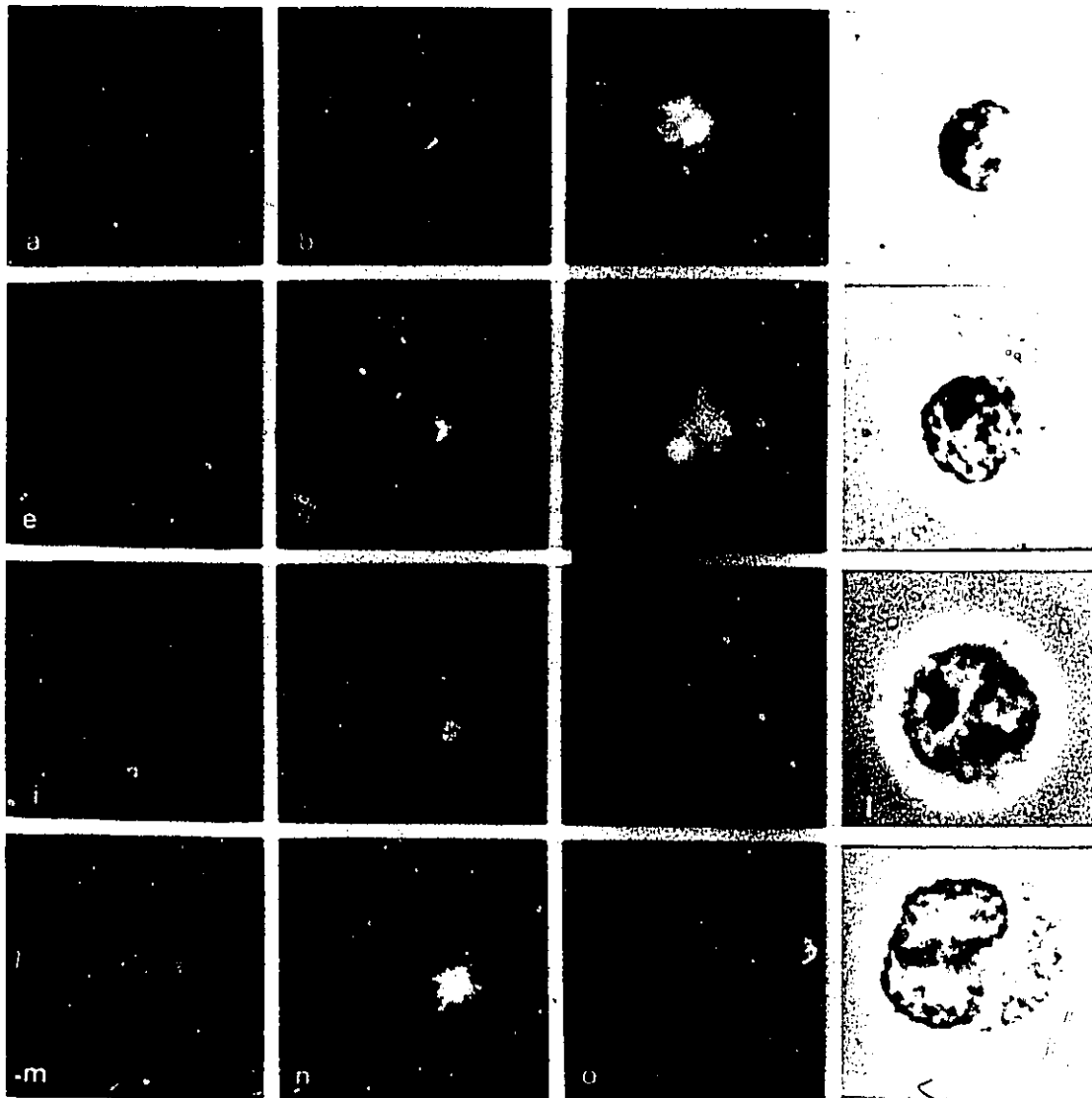
Double-immunofluorescence procedures after permeabilization and fixation of the cells (protocol II) were used to determine if a particular distribution of CaM correlated with the changes of the MT system in lymphocytes. The anti-CaM was detected with a fluorescein-labelled antibody against the anti-CaM. The anti-tubulin 1D9 was detected with a

rhodamine-labelled antibody against the anti-tubulin. Double immunofluorescence allows the detection of two different antigens within the same cell, and thus direct comparisons and correlations between the antigens are possible. Neither resting (Fig. 7a) nor partially stimulated lymphocytes at 24 hours (Fig. 7e) had CaM colocalized with the microtubular system. Resting (Fig. 7b, c, d) and partially stimulated lymphocytes of 24h (Fig. 7f, g, h) had only a few MT in their cytoplasm, and both were small in size and had small nuclei and nucleoli.

Figures 7i-l and 7m-p are representative of the fully stimulated lymphocyte at 48h after the addition of concanavalin A. The localization of CaM in 48h stimulated lymphocytes was restricted to a single spot or ring in the cytoplasm and some MT close to the centrosome (Fig. 7i, m). In all cases CaM distribution corresponded to the site from which MT were radiated (Fig. 7j, n). Phase microscopy showed that CaM was localized in the centrosomal region located in the nuclear cleft (Fig. 7l, p). The cells that showed CaM in the centrosomal region had an increased cell or nuclear sizes, large nucleoli and disaggregated chromatin (Fig. 7l, p).

Figure 7. Double immunofluorescence staining of CaM and tubulin in resting, 24h and 48h stimulated lymphocytes.

a, b, c and d resting lymphocytes; e, f, g and h 24h stimulated lymphocytes; i to p 48h stimulated lymphocytes. a, e, i and m calmodulin staining; b, f, j and n tubulin staining; c, g, k and o DNA staining; d, h, i and p phase microscopy. Cells were processed according to protocol II. Sheep anti-CaM was detected with FITC-rabbit anti-sheep IgG. Mouse anti-tubulin was detected with a TRITC-goat anti-mouse IgG. Bar = 1.0 μ m.

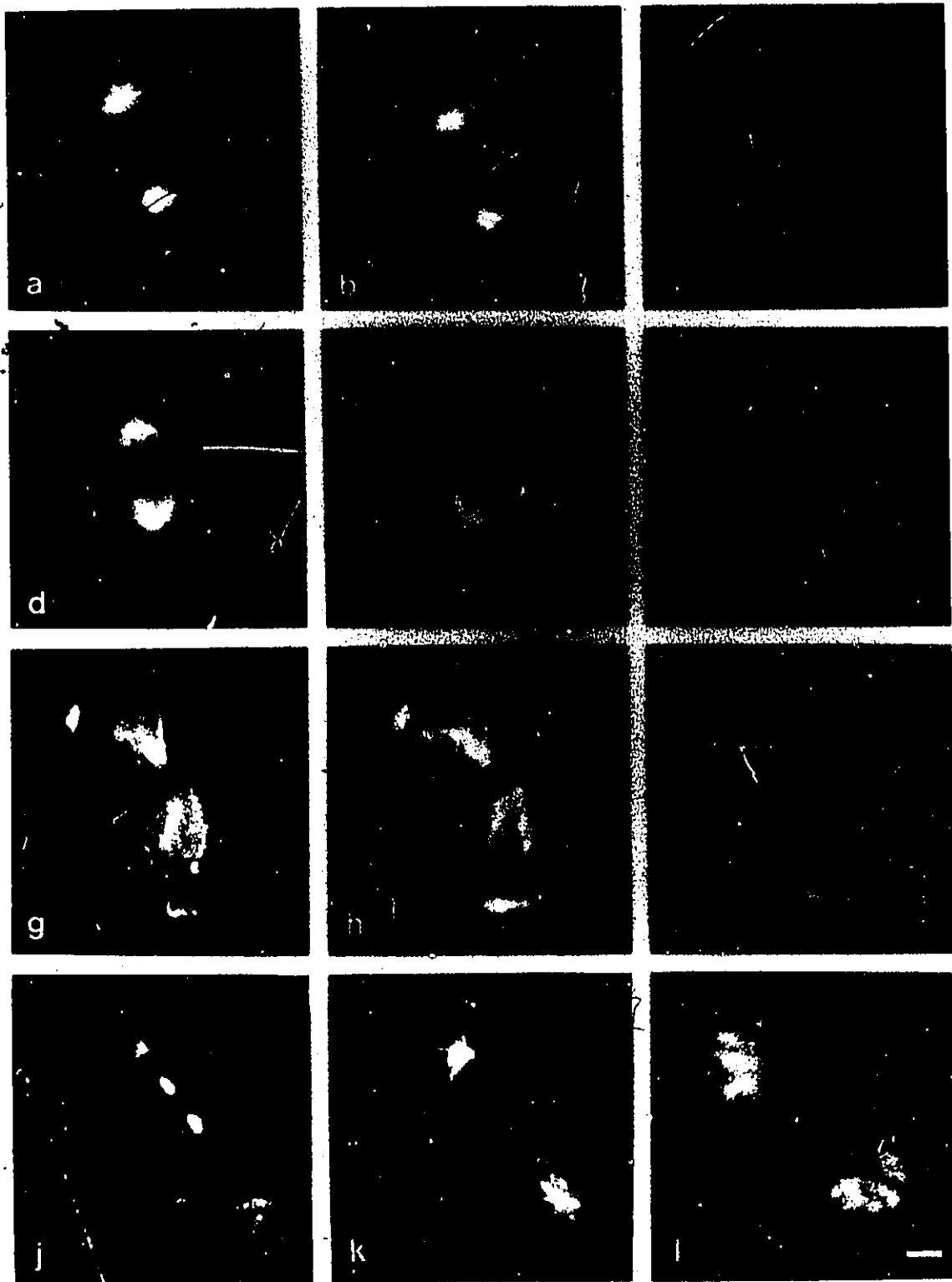


The distribution of calmodulin and microtubules during
mitosis

Double immunofluorescence staining was used to examine the distribution of CaM relative to the mitotic spindle in mitotic lymphocytes. The specific stage of mitosis in the cells examined was identified with the DNA specific stain, Hoechst 33258 (Fig. 8c, f, i, l). As shown in figure 8a and 8b, CaM remains colocalized with the centrosome and MT after the centrosome duplication and migration of each daughter centrosome to the mitotic poles. At metaphase, CaM was found in the half spindles and mitotic poles at both sides of the chromosomal plate (Fig. 8d). Tubulin showed a more extended staining across the mitotic spindle at metaphase (Fig. 8e). In early anaphase, CaM still remained in the polar region; however, during late anaphase, in addition to its localization at the poles, CaM was also localized with the MT appearing between the two masses of daughter chromosomes (Fig. 8g, h). During telophase and cytokinesis, CaM was localized with the remaining bundles of MT at both sides of the midbody and the centrosomes of the daughter cells (Fig. 8j, k).

Figure 8. Double immunofluorescence staining of CaM and tubulin in lymphocytes during mitosis.

a, b and c prophase; d, e and f metaphase; g, h and i anaphase; j, k, and l telophase. a, d, g and j CaM staining; b, e, h and k tubulin staining; c, f, i and l DNA staining. Mouse lymphocytes were stimulated with con A for 48h and then processed according to protocol II. Sheep anti-CaM was detected with a FITC-rabbit anti-sheep IgG. Mouse anti-tubulin was detected with a TRITC-goat anti-mouse IgG. Bar = 1.0 um.



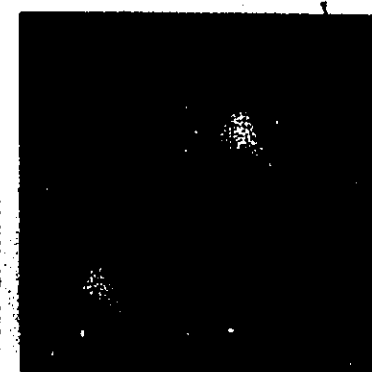
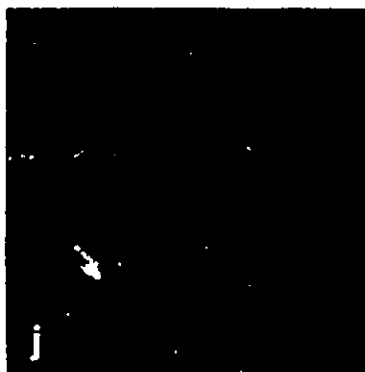
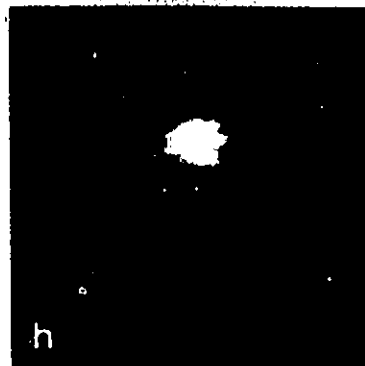
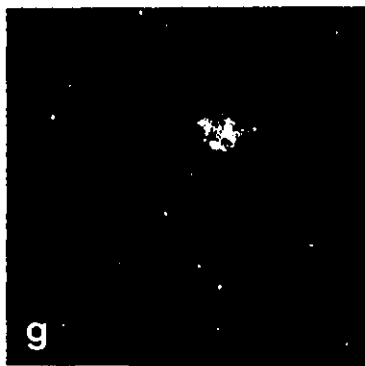
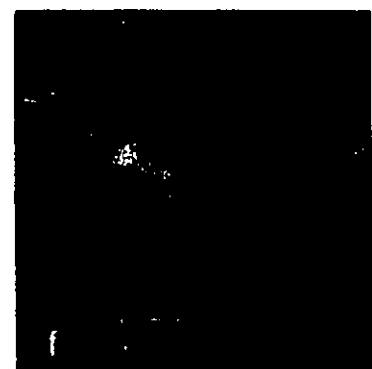
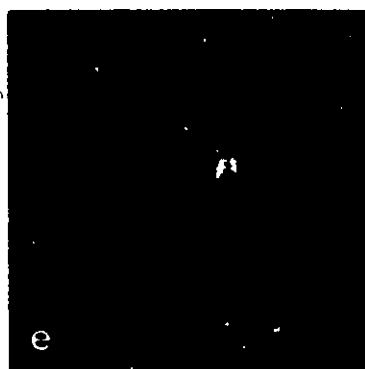
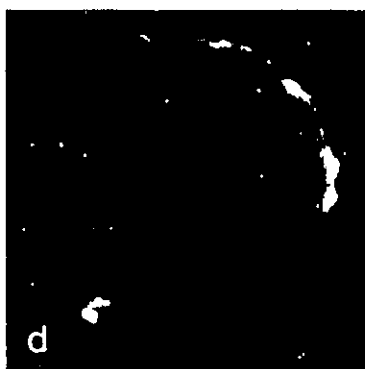
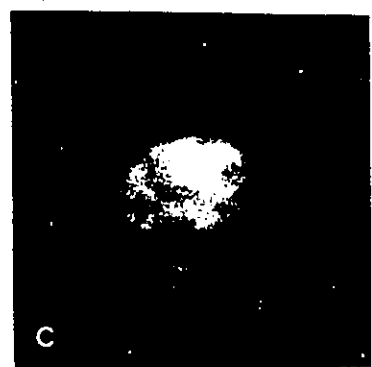
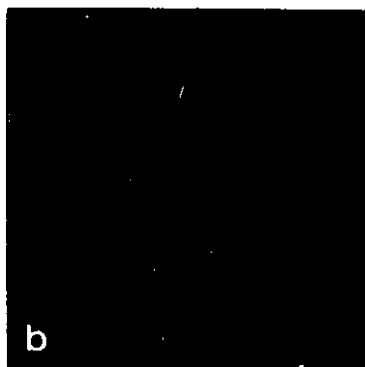
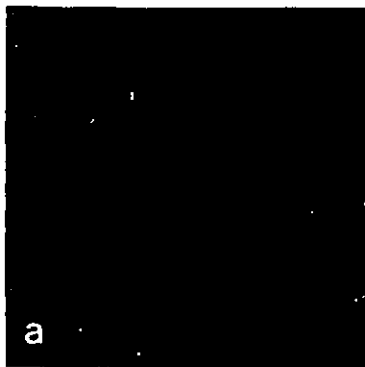
The effects of 1.2 mM CaCl_2 and 10 mM EGTA on the
distribution of CaM and microtubules

Previous studies have shown that some CaM interactions with the cytoplasmic matrix are calcium-independent. Double immunofluorescence was used to examine the effects of an excess of calcium or its absence on the localization of CaM and its relationship to the microtubular system in 48h stimulated lymphocytes.

If no EGTA was included during the Triton X-100 permeabilization and fixation steps, the solutions contained unbuffered 1.2 mM CaCl_2 . Under this EGTA-free condition, CaM was found diffusely distributed in the cytoplasm of 48h stimulated lymphocytes (Fig. 9a) and no MT were visible (Fig. 9b). Mitotic cells treated with EGTA-free buffers still had some CaM localized in the mitotic spindle (Fig. 9d), and this corresponded to the remaining Ca^{2+} -resistant spindle MT (Fig. 9e). Those cells also had an additional CaM staining near the membrane (Fig. 9d). The exclusion of CaCl_2 and addition of 10 mM EGTA in all washing, permeabilization and fixation buffers, preserved the CaM localization in the centrosome of stimulated cells (Fig. 9g). The same cell still had an intact microtubular system (Fig. 9h). Mitotic cells in 10 mM EGTA buffers still had CaM localized in the mitotic poles (Fig. 9j) and a well preserved mitotic spindle (Fig. 9h).

Figure 9. Double immunofluorescence staining of CaM and tubulin in 1.2 mM CaCl_2 or 10 mM EGTA-treated lymphocytes.

Cells were processed according to protocol II, either in the absence of EGTA (a, b, c, d, e and f) or in the absence of CaCl_2 but the presence of 10 mM EGTA (g, h, i, j, k and l). a, b, c, g, h and i are lymphocytes at interphase; d, e, f, j, k and l are lymphocytes during mitosis. a, d, g and j are CaM staining; b, e, h and k tubulin staining; and c, f, i and l DNA staining. CaM antibody was detected with an FITC-labelled anti-sheep IgG, and tubulin antibody was detected with a TRITC-labelled goat anti-mouse IgG. Bar = 1 μm .



The effects of colchicine, colcemid and cold on the
distribution of calmodulin

It has been previously demonstrated that colchicine disassembled MT but did not cause any major disturbances on the organization of the centrosome (119). However, cold-induced disassembly of MT altered the centrosomal region in a reversible manner. Therefore, double immunofluorescence was used to study the effects of the anti-microtubular drugs, colchicine and colcemid, and also cold temperature on the distribution of CaM in the microtubular system. 48h stimulated lymphocytes were incubated for 1 hour in the presence of 1 ug/ml colchicine or colcemid, and then the cells were processed according to protocol II. Calmodulin was found localized in a restricted region in the cytoplasm of colchicine (Fig. 10a) or colcemid (Fig. 10d) treated lymphocytes. The distribution of CaM coincided with the colchicine (Fig. 10b) and colcemid (Fig. 10e) resistant staining of tubulin in the centrosomal region.

In this particular study, washings and permeabilization steps before the fixation of the cells were done on ice. Thirty minutes cold treatment (0°C - 4°C) of interphase (Fig. 11a) or mitotic (Fig. 11d) lymphocytes resulted in a diffuse localization of CaM and no MT (Fig. 11b, e), respectively. When cold-treated cells were transferred back to 37°C for 2 min (Fig. 11h) or 5 min (Fig. 11k, n), microtubules were

Figure 10. Double immunofluorescence staining of CaM and tubulin in colchicine or colcemid-treated lymphocytes.

48h stimulated lymphocytes were treated with 1 ug/ml colchicine or colcemid for 1h, and processed according to protocol II. a, b, and c are a colchicine-treated lymphocyte; d, e and f colcemid-treated lymphocytes; a and d CaM staining; b and e tubulin staining; and c and f DNA staining. Anti-CaM was detected with an FITC-labelled anti-sheep IgG. Anti-tubulin was detected with a TRITC-labelled anti-mouse IgG. Bar = 1 um.

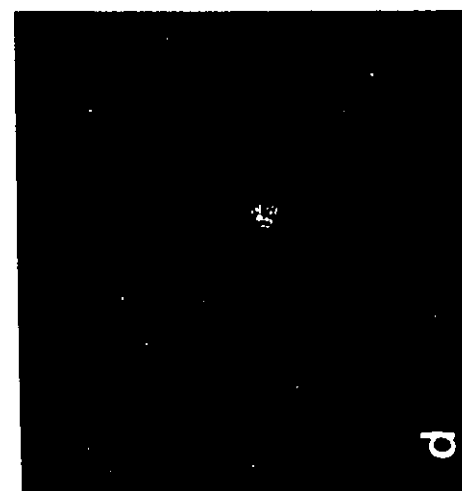
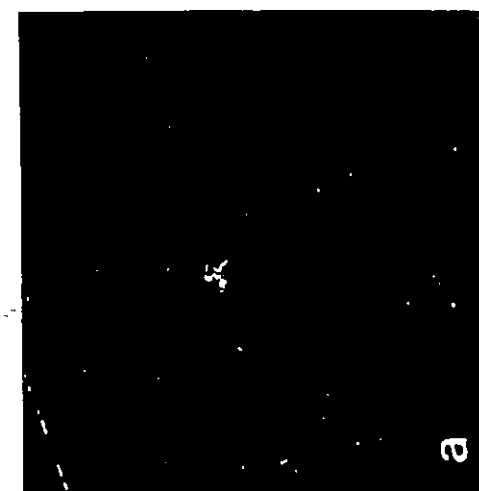
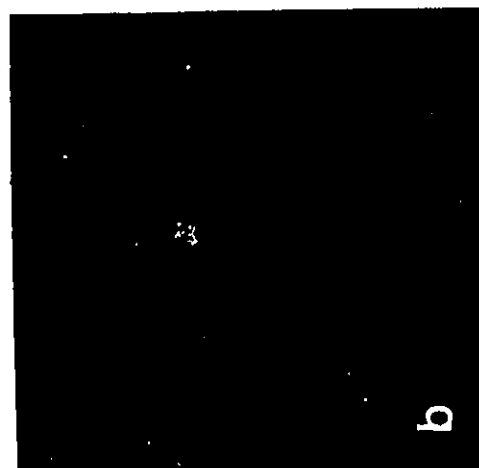
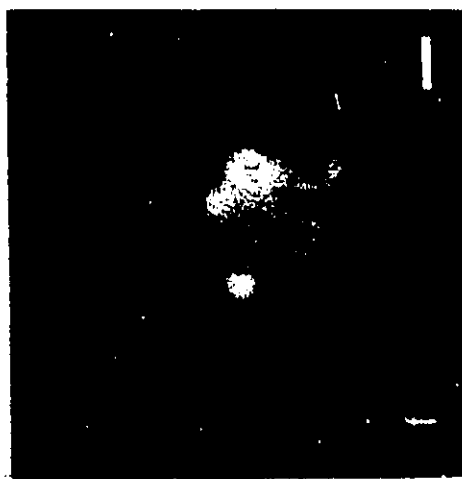
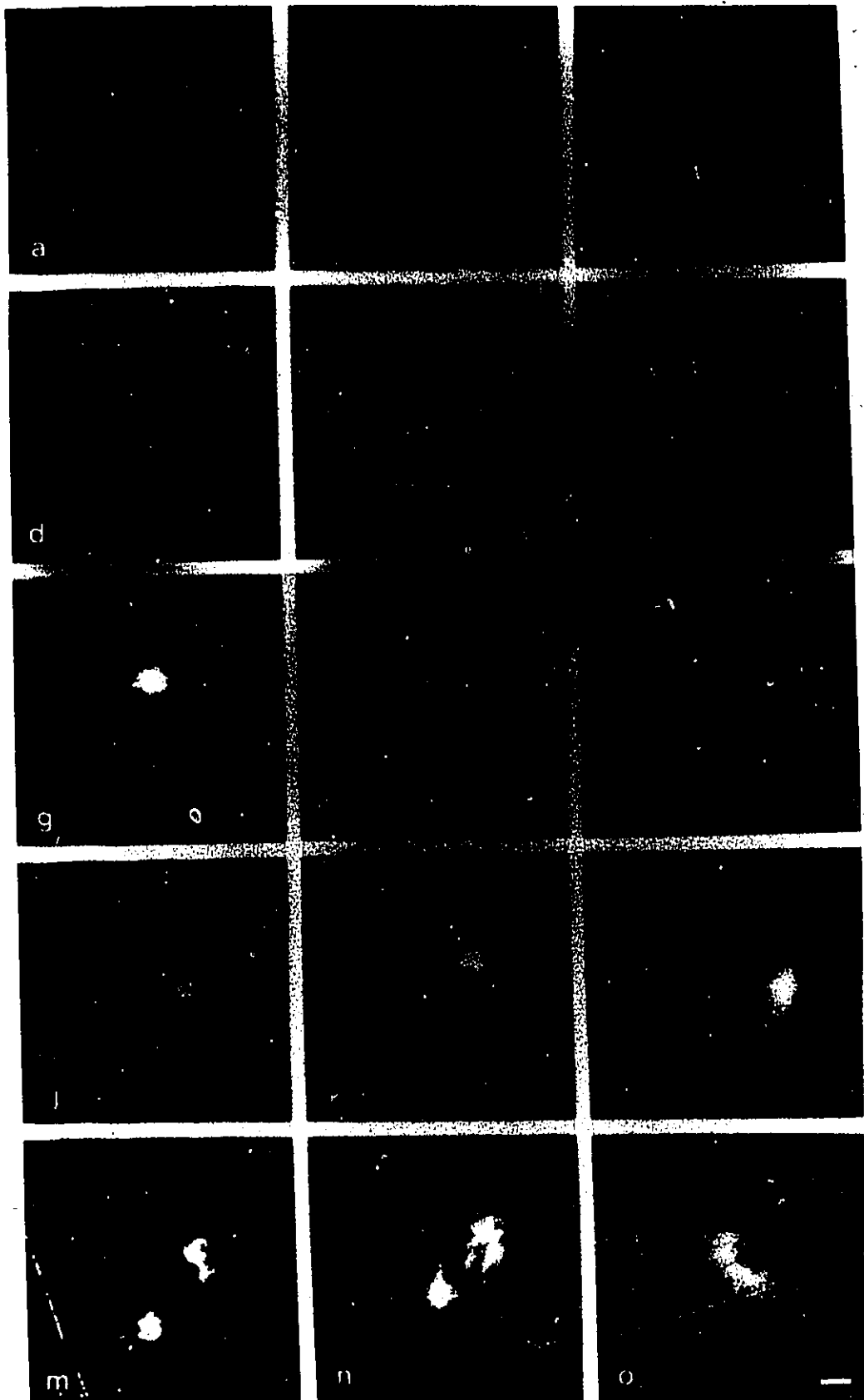


Figure 11. Double immunofluorescence staining of CaM and tubulin in cold-treated lymphocytes.

48h stimulated and mitotic lymphocytes were exposed to cold for 30 min and then processed as indicated for protocol II (a, b, c, d, e and f), or alternatively rewarmed at 37°C for 2 min (g, h and i) or 5 min (j, k, l, m, n and o) before permeabilization and fixation. a, b, c, g, h, i, j, k and l are interphase lymphocytes; d, e, f, m, n and o mitotic lymphocytes; a, d, g, j and m CaM staining; b, e, h, l and n tubulin staining; and c, f, i, l and o DNA staining. Anti-CaM was detected with an FITC-labelled anti-sheep IgG. Anti-tubulin was detected with a TRITC-labelled anti-mouse IgG. Bar = 1.0 μ m.



reassembled from centrosomes or mitotic poles, and CaM was localized on those centrosomes and some MT (Fig. 11g, j, m).

The effects of taxol on the distribution of calmodulin

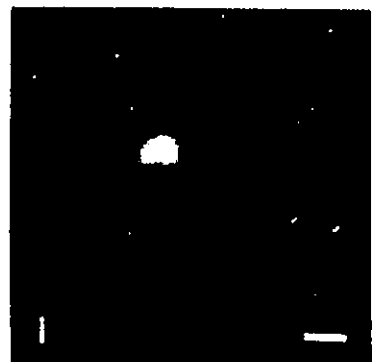
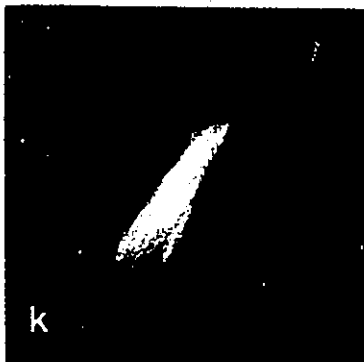
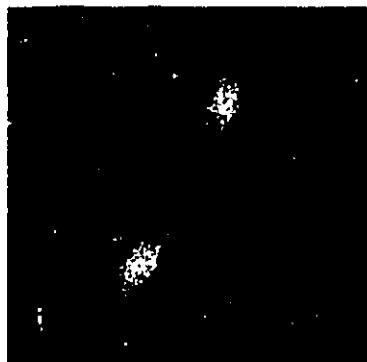
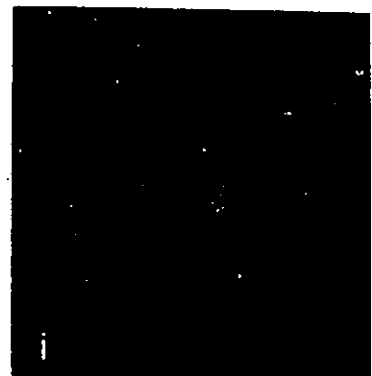
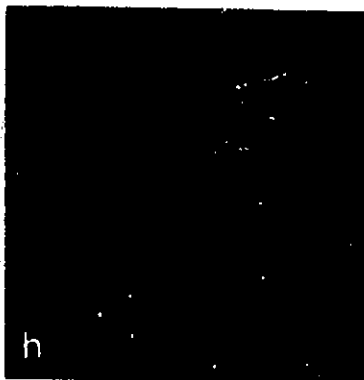
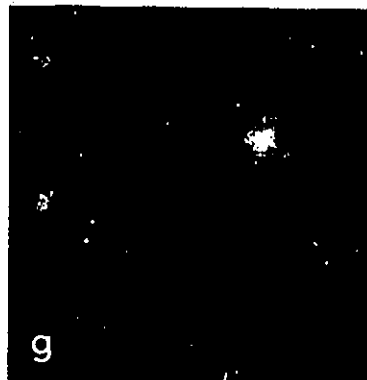
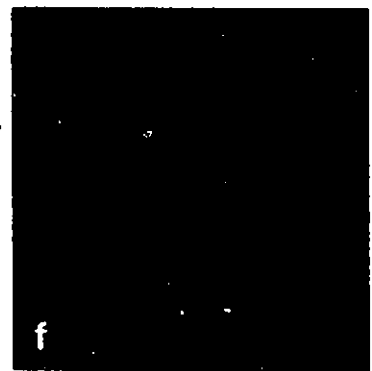
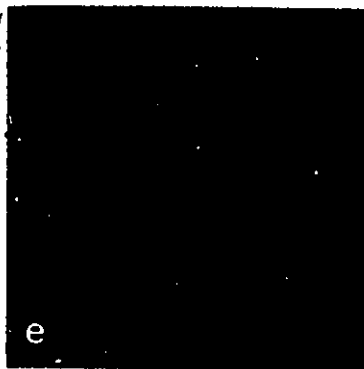
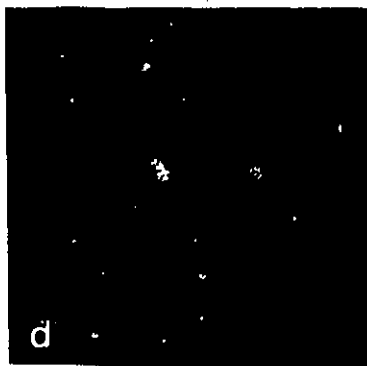
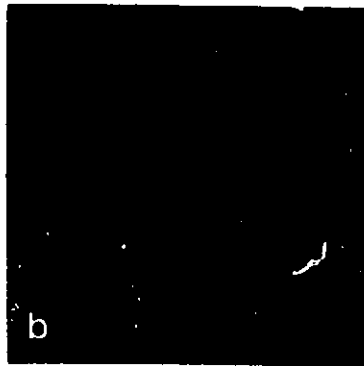
The microtubule assembly promoting drug taxol, induces the formation of bundles of MT from the interphase centrosome and multiple asters during mitosis in mouse splenic lymphocytes (152). Therefore, the distribution of CaM and MT in taxol-treated cells that were processed with protocol II, was examined by double immunofluorescence.

48h stimulated lymphocytes treated with 10 μ M taxol during 1 hour had bundles of MT in interphase (Fig. 12b) and multiple asters at mitosis (Fig. 12c). CaM was colocalized with the taxol-induced bundles of MT and multiple asters (Fig. 12a, d).

Calmodulin in stimulated cells treated with 10 μ M taxol during 48h (Fig. 12g) was still colocalized on the bundles of MT (Fig. 12h). The CaM localization (Fig. 12j) in the 10 μ M taxol-induced bundles of MT (Fig. 12k) was unaltered when EGTA-free buffers (1.2mM CaCl_2) were used in protocol II:-

Figure 12. Double immunofluorescence staining of CaM and tubulin in taxol-treated lymphocytes.

48h stimulated lymphocytes were treated with 10 μ M taxol for 1h (a, b, c, d, e and f) or 48h (g, h, i, j, k and l). Cells were processed according to protocol II in the presence of 1.2 mM CaCl_2 + 2 mM EGTA (a, b, c, d, e, f, g, h and i) or 1.2 mM CaCl_2 with no EGTA (j, k and l). CaM staining was a, d, g and j; tubulin staining was b, e, h and k; and DNA staining was c, f, i and l. Anti-CaM was detected with an FITC-labelled anti-sheep IgG. Anti-tubulin was detected with a TRITC-labelled anti-mouse IgG. Bar = 1.0 μ m.



The effects of calmodulin antagonists on microtubules

48h stimulated cells showed a CaM-centrosome interaction that coincided with an increased assembly of MT from the centrosome. Further, the CaM-centrosome interaction correlated with the reassembly of MT after cold treatment, suggesting a role of CaM in the nucleation or assembly of MT from the centrosome. Immunofluorescence was used to examine the effect of several CaM antagonists on the assembled MT and during their reassembly after cold treatment.

In this particular study the cells were incubated in the presence of CaM antagonists in serum-free DMEM media since those drugs are known to bind to serum proteins. Indirect immunofluorescence was done in cells first fixed in aldehydes and subsequently permeabilized with Triton X-100 (protocol III, see Materials and Methods). This was done to avoid displacement of the CaM antagonists by Triton X-100 (before fixation), which might have resulted in ambiguous interpretation.

Table II summarizes the effects of the different antagonists and concentrations used on the viability and MT of 48h stimulated cells at 37°C. Doses up to 25 μ M of both W-7 and W-5, and 10 μ M of both TFP and CDZ had little effect on the viability of 48h stimulated T cells over a period of 75 min.

TABLE II. Effect of CaM antagonists on MT of 48h stimulated lymphocytes.

TREATMENT		% VIABLE LYMPHOCYTES	CELLS WITH MT (%) TOTAL LYMPHOCYTES
-	37°C	92	92
W-7 (1 uM)	"	96.8	89.1
W-7 (10 uM)	"	94.6	88.5
W-7 (25 uM)	"	71.4	70.8
W-5 (1 uM)	"	96.8	93.0
W-5 (10 uM)	"	94.9	95.1
W-5 (25 uM)	"	86.9	91.2
TFP (1 uM)	"	95.5	75.8
TFP (10 uM)	"	96.8	66.5
CDZ (1 uM)	"	94.7	90.1
CDZ (10 uM)	"	72.5	78.4

Two ml of 48h stimulated cells were exposed to each drug concentration for 75 min, and then an aliquot was used to examine cell viability by the trypan blue exclusion test. Two hundred cells of each treatment were examined. At 65 min of the drug treatment, 200 ul of cells were attached to half coverslips for 10 min and then processed according to protocol III for tubulin staining. Two hundred cells of each drug treatment were examined. This table represents the mean of two different experiments.

Forty-eight hours stimulated lymphocytes had intact MT radiating from a common point (Fig. 13a, b). The microtubular system of lymphocytes remained unaffected when the cells were treated at 37°C for 70 min with doses up to 25 μ M W-7 (Fig. 13c, d), 25 μ M W-5 (Fig. 13e, f), 10 μ M TFP (Fig. 13 g, h) and 10 μ M CDZ (Fig. 13i, j).

Table III summarizes the effects of different doses of CaM antagonists during the reassembly of MT from cold treatment in 48h stimulated lymphocytes.

When cells were incubated for 30 min at 0° - 4°C, there was no visible MT or defined tubulin staining in interphase lymphocytes (Fig. 14a, b). When cold-treated cells were rewarmed for 10 min at 37°C, MT reassembly from the centrosome was detected (Fig. 14c, d). The reassembly of MT from the centrosome was then examined in cells incubated with the different CaM antagonists for 70 min (30 min before cold treatment (37°C), 30 min of cold treatment (0° - 4°C), 10 min reassembly at 37°C). Twenty-five μ M W-7 inhibited the reassembly of MT (Fig. 14e, f). However, when 25 μ M W-5 (the less active analog of W-7) was present during the disassembly and rewarming, MT were detected radiating from the centrosome (Fig. 14g, h). Cells treated with 10 μ M TFP (Fig. 14 i, j) or 10 μ M CDZ (Fig. 14k, l) did not have visible MT after the cold exposure and rewarming at 37°C.

Figure 13. Immunofluorescence staining of tubulin in 48h stimulated lymphocytes treated with CaM antagonists.

Cells were treated with no antagonists (a, b) or were incubated at 37°C for 75 min with 25 μ M W-7 (c, d); 25 μ M W-5 (e, f); 10 μ M TFP (g, h); and 10 μ M CDZ (i, j). a, c, e, g and i are tubulin staining; b, d, f, h and j are DNA staining. After treatments, cells were processed according to protocol III. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μ m.

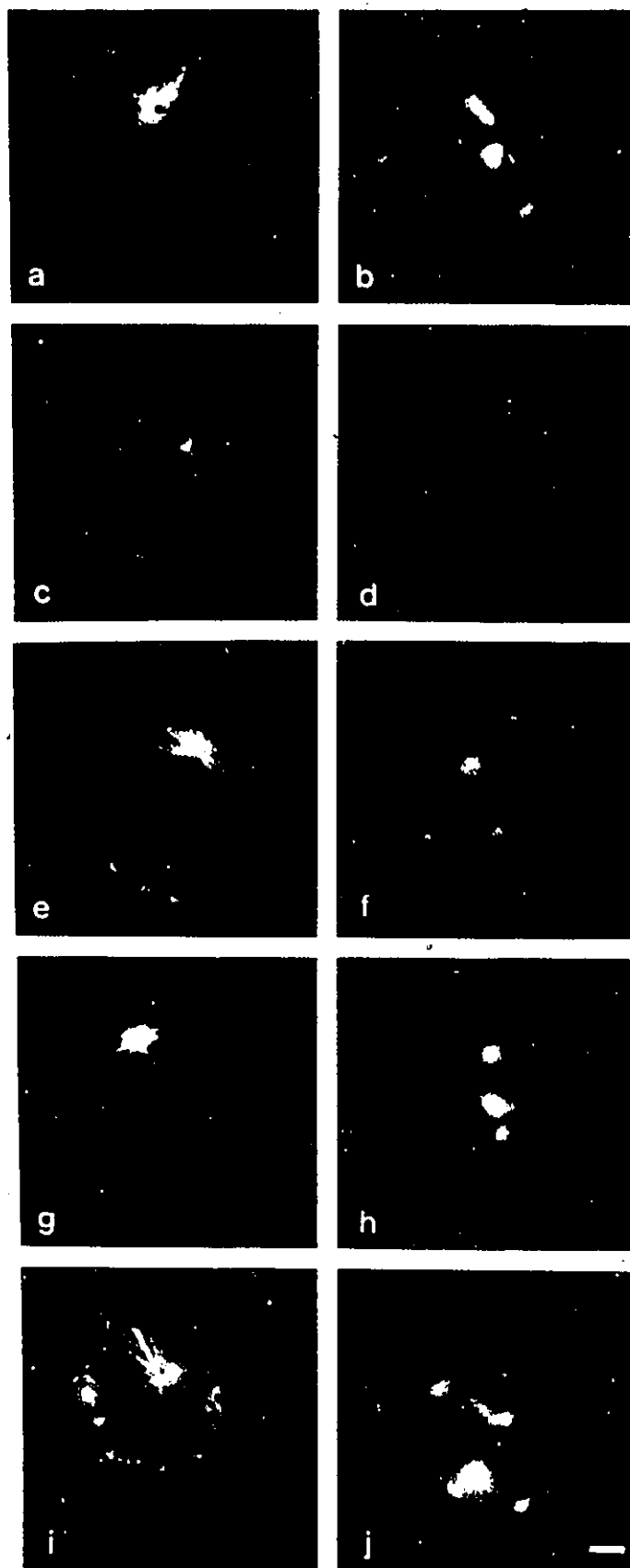


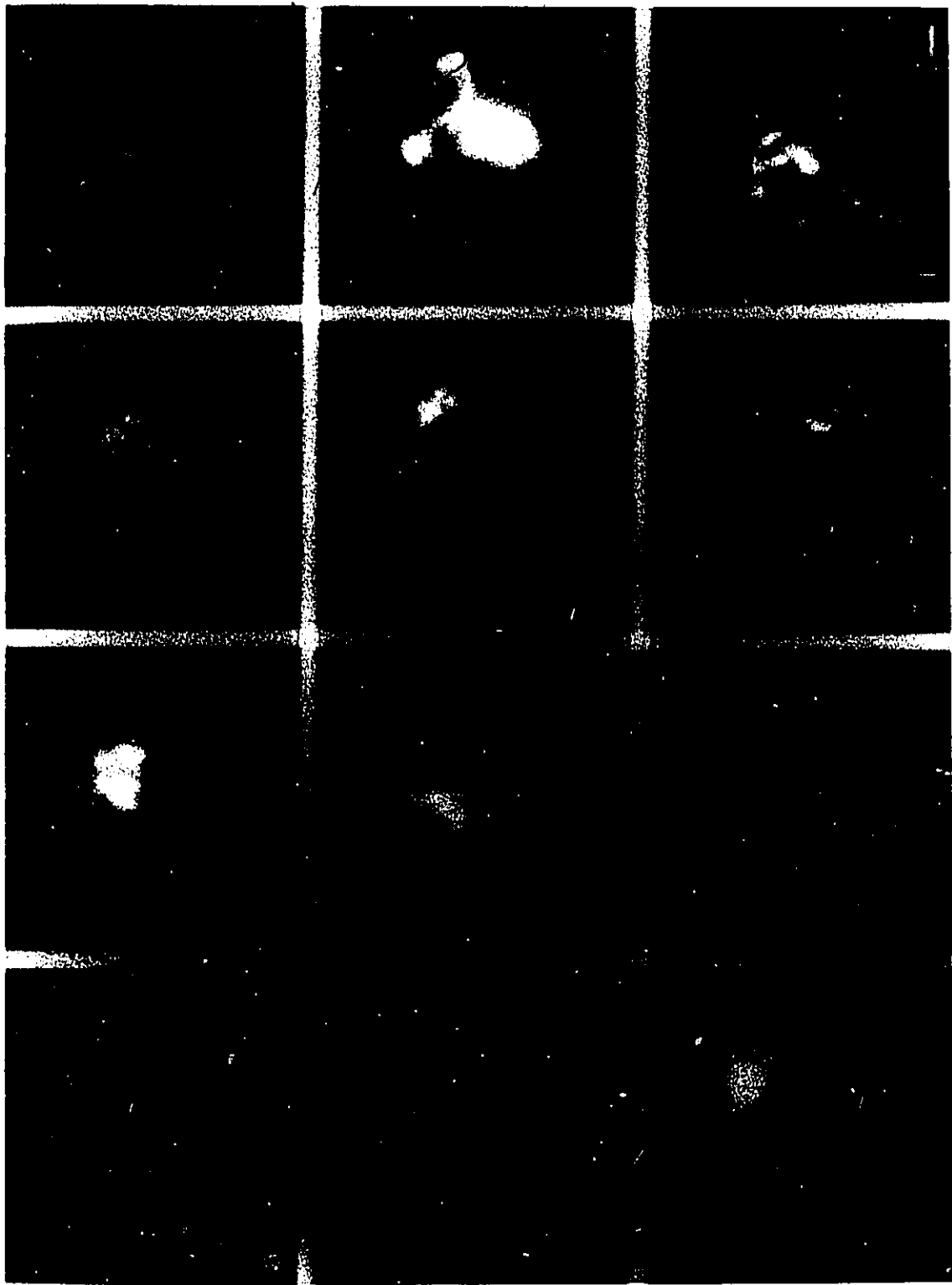
TABLE III. Effect of CaM antagonists on the reassembly of MT from cold in 48h stimulated lymphocytes.

COLD TREATMENT		% VIABLE LYMPHOCYTES	CELLS WITH MT (%) TOTAL LYMPHOCYTES
No drug		88.9	0.0
No drug	+ Reassembly	84.8	81.8
W-7 (1 uM)	"	86.1	85.2
W-7 (10 uM)	"	87.1	89.4
W-7 (25 uM)	"	90.1	26.2
W-5 (1 uM)	"	86.8	92.5
W-5 (10 uM)	"	89.4	90.8
W-5 (25 uM)	"	90.5	79.3
TFP (7 uM)	"	88.3	77.2
TFP (10 uM)	"	97.6	6.3
CDZ (1 uM)	"	84.2	92.6
CDZ (10 uM)	"	72.1	24.3

Two ml of 48h stimulated cells were exposed to each drug concentration for 30 min at 37°C, and then for 30 min at 4°C, and then they were incubated at 37°C for 10 min. An aliquot of each drug treatment was used to determine cell viability by the trypan blue exclusion test. Two hundred cells of each treatment were examined. At 60 min of the drug treatment, 200 ul of cells were attached to half coverslips for 10 min and then processed according to protocol III for tubulin staining. Two hundred cells of each drug treatment were examined. This table represents the mean of two different experiments.

Figure 14. Immunofluorescence staining of tubulin in 48h stimulated lymphocytes treated with CaM antagonists during the cold-disassembly and reassembly of MT.

A cell was exposed to 4°C for 30 min (a, b). A cell was exposed to 4°C for 30 min and then rewarmed at 37°C for 10 min (c, d). Cells exposed to 25 μ M W-7 (e, f); 25 μ M W-5 (g, h) 10 μ M TFP (i, j) and 10 μ M CDZ (k, l) for 30 min at 37°C, followed by 30 min at 4°C, were then rewarmed at 37°C for 10 min. After treatments, cells were processed according to protocol III. Tubulin staining was a, c, e, g, i and k; and DNA staining was b, d, f, h, j and l. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μ m.



The effects of calcium on the stability of microtubules

Calmodulin has been found ~~co~~localized in the centrosomal region and some MT in stimulated lymphocytes and 3T3 cells (96), and closely associated with the mitotic spindle during mitosis. If CaM induces the disassembly of MT, it should depend upon the formation of calcicalmodulin complexes. Therefore, the effects of increasing calcium concentrations in the microtubular system were examined in stimulated lymphocytes and 3T3 cells. Free calcium concentrations between 0.1 μM and 50 μM were achieved with calcium-EGTA buffers. Above 50 μM Ca^{2+} , the calcium-EGTA buffer capacity is weak and therefore 0.1 mM and 1.0 mM Ca^{2+} were achieved by direct addition of the proper amount of a 0.1M stock solution of CaCl_2 . The rationale was to gently permeabilize the cells with Brij-58 to minimize the loss of cellular components and then allow those "cells" to equilibrate for 10 min with the desired calcium concentration of the bathing solution. Thereafter, the fixation step was used to preserve the microtubular system after the different treatments. Indirect immunofluorescence with the anti-tubulin 1D9 was used to examine the calcium effects on the microtubular system (see protocol IV, Materials and Methods).

Stimulated mouse T lymphocytes and 3T3 cells both during interphase and mitosis exposed to Ca^{2+} concentrations between <.01 μM to 50 μM , had an intact microtubular system (Fig. 15a

to t, lymphocytes; Fig. 16a to e, interphase 3T3 cells; and Fig. 17 a to j, mitotic 3T3 cells).

Stimulated lymphocytes in interphase, treated with 0.1 mM Ca^{2+} (Fig. 15u, v) or 1.0 mM Ca^{2+} (Fig. 15y, z), only had a spot of tubulin staining over the centrosome. Mitotic lymphocytes exposed to 0.1 μM Ca^{2+} (Fig. 15w, x) or 1.0 mM Ca^{2+} (Fig. 15a', b') still had a visible tubulin staining on the mitotic spindle. Tubulin staining of interphase 3T3 cells treated with 0.1 mM Ca^{2+} (Fig. 16f) or 1.0 mM Ca^{2+} (Fig. 16g) showed pieces of MT scattered through the cytoplasm and most frequently found closed to the centrosome. 3T3 cells at mitosis treated with 0.1 mM Ca^{2+} still had a visible tubulin staining in the mitotic spindle (Fig. 17k). Mitotic 3T3 cells treated with 1.0 mM Ca^{2+} still had fragments of MT very close to the mitotic poles (Fig. 17m).

The possibility for the calcium disassembled MT to reassemble was examined next. Brij-58 extracted cells that had their MT previously disassembled by cold exposure (Fig. 18a, b) still had the capacity to form a normal microtubular system if the cold-treated and extracted cells were transferred to a reassembly buffer at 37°C (Fig. 18c, d). However, neither cells exposed to 0.1 mM Ca^{2+} (Fig. 18 e, f) nor 1.0 mM Ca^{2+} (Fig. 18g, h) was able to reform a microtubular system when the same cells were transferred to the reassembly buffer (1.0 μM Ca^{2+}) at 37°C for 15 min,

Figure 15. Immunofluorescence staining of tubulin in Brij-58 permeabilized lymphocytes exposed to different calcium concentrations.

48 h stimulated lymphocytes in interphase and mitosis were processed according to protocol IV and exposed to different calcium concentrations: $< 0.01 \mu\text{M Ca}^{2+}$ (a, b, c and d); $0.1 \mu\text{M Ca}^{2+}$ (e, f, g and h); $1.0 \mu\text{M Ca}^{2+}$ (i, j, k and l); $10 \mu\text{M Ca}^{2+}$ (m, n, o and p); $50 \mu\text{M Ca}^{2+}$ (q, r, s and t); 0.1 mM Ca^{2+} (u, v, w and x); and 1.0 mM Ca^{2+} (y, z, a' and b'). Tubulin staining is shown in a, c, e, g, i, k, m, o, q, s, u, w, y and a'; and DNA staining in b, d, f, h, j, l, n, p, r, t, v, x, z and b'. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = $1.0 \mu\text{m}$.

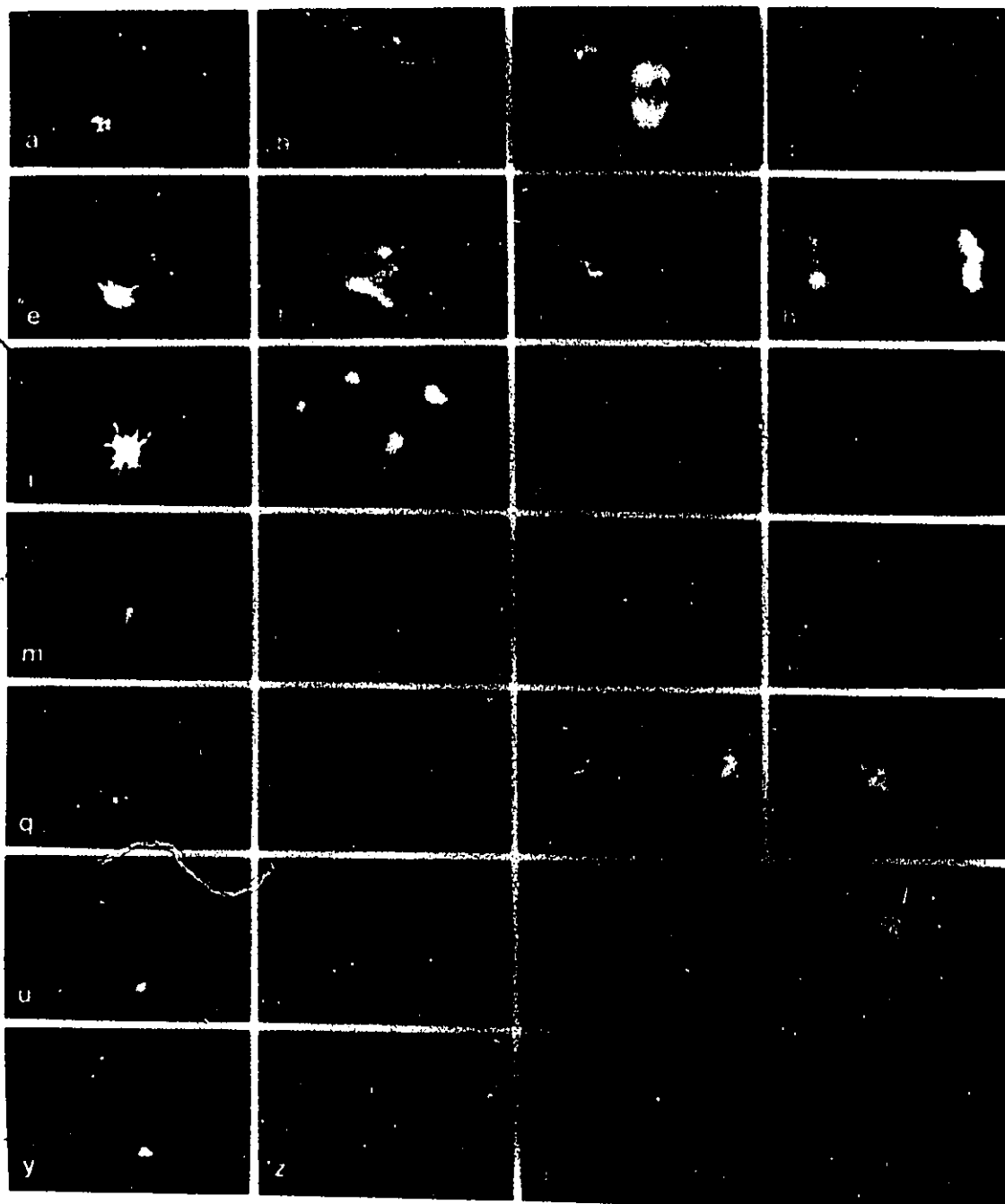


Figure 16. Immunofluorescence staining of tubulin in Brij-58 permeabilized 3T3 cells at interphase, exposed to different Ca^{2+} concentrations.

Interphase 3T3 cells were processed according to protocol IV and exposed to different calcium concentrations: $< 0.01 \text{ uM } \text{Ca}^{2+}$ (a); $0.1 \text{ uM } \text{Ca}^{2+}$ (b); $1.0 \text{ uM } \text{Ca}^{2+}$ (c), $10 \text{ uM } \text{Ca}^{2+}$ (d); $50 \text{ uM } \text{Ca}^{2+}$ (e); $0.1 \text{ mM } \text{Ca}^{2+}$ (f); and $1.0 \text{ mM } \text{Ca}^{2+}$ (g).

Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 um .

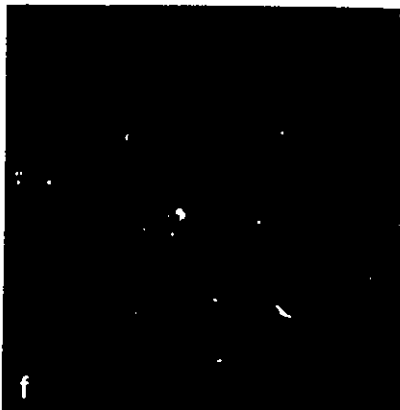
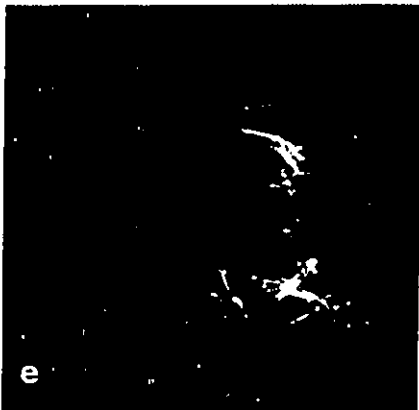
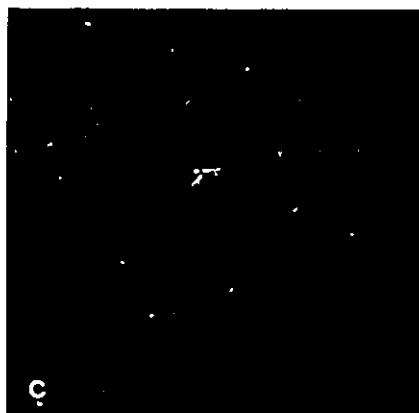


Figure 17. Immunofluorescence staining of tubulin in Brij-58 permeabilized 3T3 cells at mitosis, exposed to different Ca^{2+} concentrations.

Mitotic cells were processed according to protocol IV and exposed to different calcium concentrations: $< 0.01 \text{ uM Ca}^{2+}$ (a, b); 0.1 uM Ca^{2+} (c, d); 1.0 uM Ca^{2+} (e, f); 10 uM Ca^{2+} (g, h); 50 uM Ca^{2+} (i, j); 0.1 mM Ca^{2+} (k, l); and 1.0 mM Ca^{2+} (m, n). Tubulin staining is shown in a, c, e, g, i, k and m; and DNA staining in b, d, f, h, j, l and n. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 um .

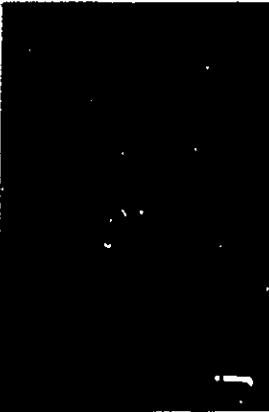
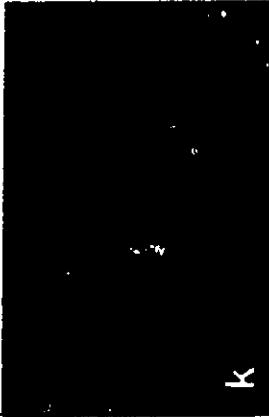
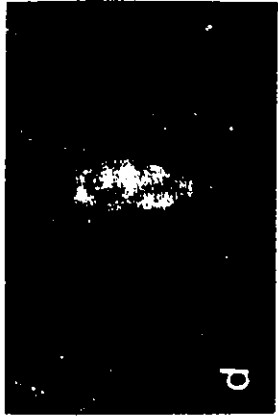
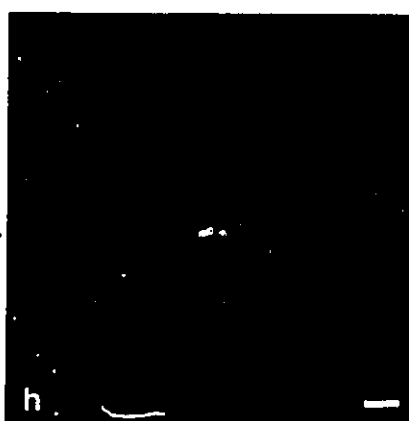
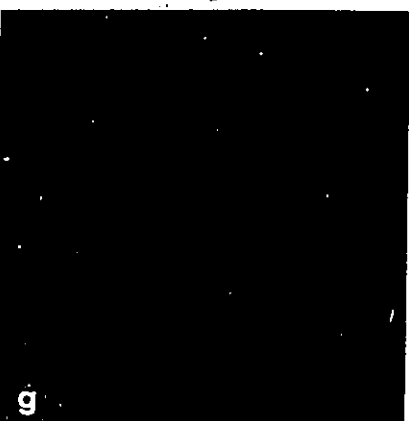
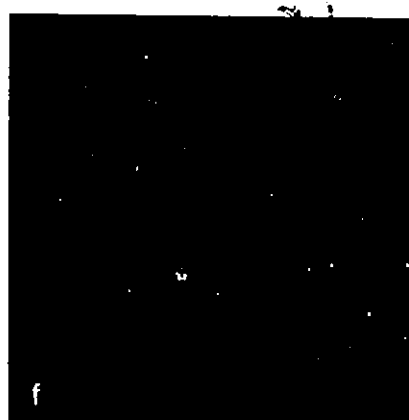
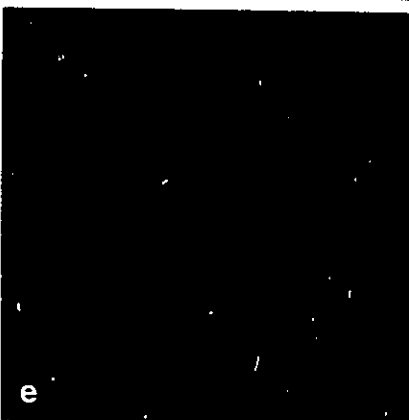
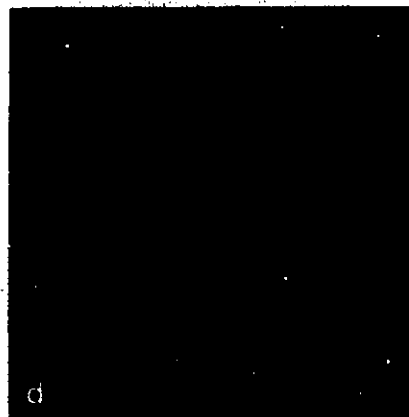
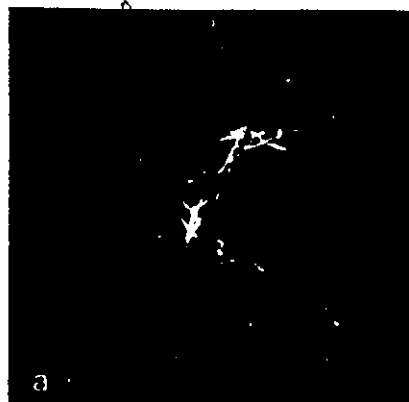


Figure 18. Immunofluorescence staining of tubulin and the irreversibility of the calcium-induced disassembly of MT.

Normal cells or cells exposed to 0°C for 30 min were processed according to protocol IV, exposed to different calcium concentrations, and then put in an R.B. (1.0 μM Ca^{2+}) at 37°C for 15 min before fixation. Cold-treated cells were exposed to 1.0 μM Ca^{2+} without a rewarming period (a, b). Cold-treated cells were exposed to 1.0 μM Ca^{2+} and rewarmed at 37°C (c, d). Normal cells were exposed to 0.1 μM Ca^{2+} (e, f) or 1.0 mM Ca^{2+} (g, h) and then put in the R.B. at 37°C for 15 min before fixation. Tubulin staining is shown in a, c, e and g; and DNA staining in b, d, f and h. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .



therefore indicating that the calcium-induced disassembly of MT is irreversible.

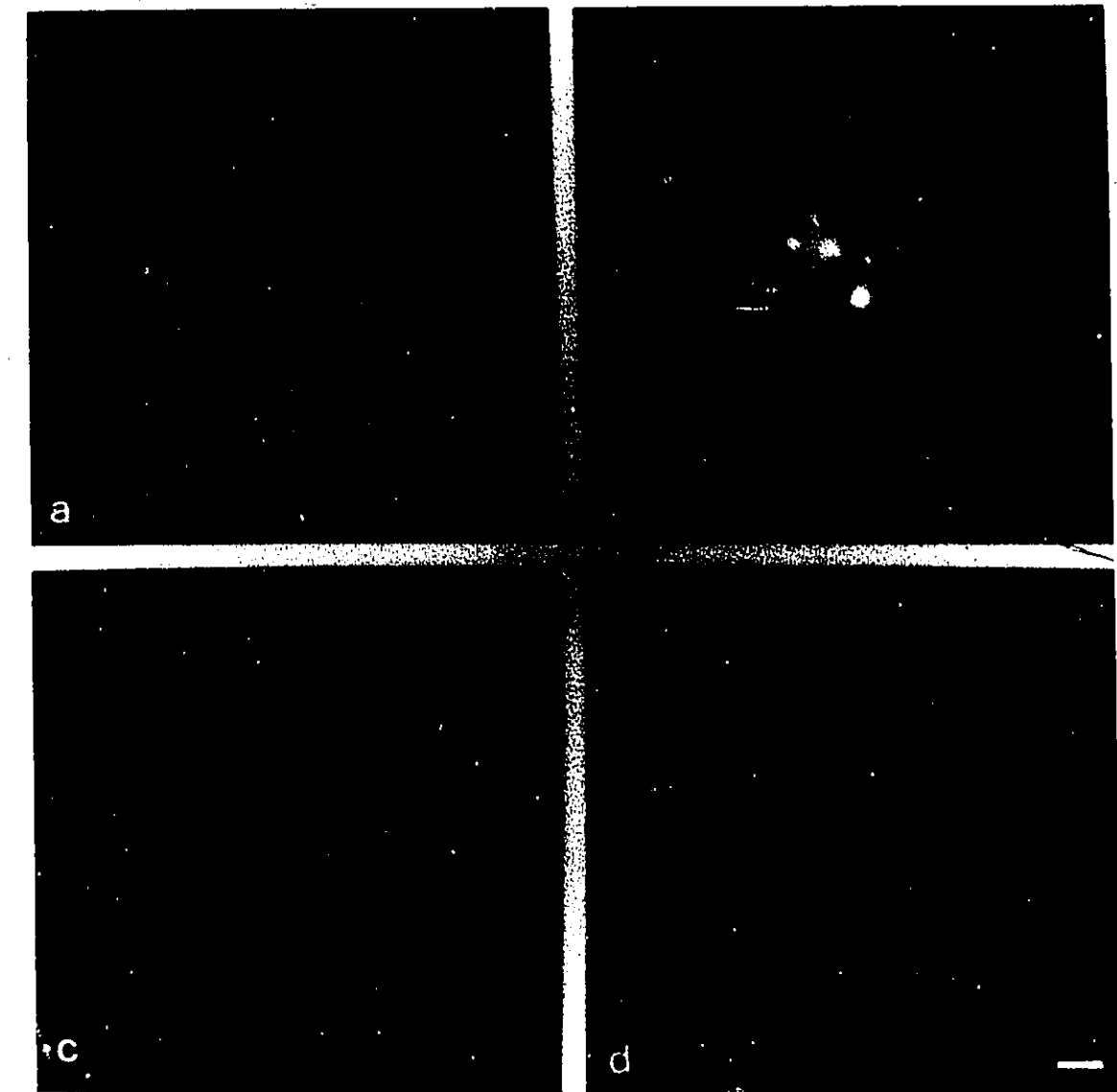
The effects of CaM antagonists and protease inhibitors on the calcium-induced disassembly of microtubules

Since it is widely believed that CaM mediates the calcium-induced disassembly of MT, the inclusion of CaM antagonists might give an indication of the role of CaM activity during the disassembly of MT. In this study, CaM antagonists were included at all steps. Cells were first equilibrated and permeabilized (protocol IV) in a buffer containing $1.0 \mu\text{M Ca}^{2+}$ in the presence of the CaM antagonists. Then, cells were equilibrated for 10 min in 0.1 mM Ca^{2+} , also in the presence of CaM antagonists. The cells were then fixed in a buffer containing 0.1 mM calcium with no antagonists. Indirect immunofluorescence was used to examine the combined effects of CaM antagonists and 0.1 mM Ca^{2+} on the microtubular system of 3T3 cells. Neither $10 \mu\text{M}$ W-7 (Fig. 19a, b) nor $20 \mu\text{M}$ TFP (Fig. 19c, d) affected the 0.1 mM calcium-induced disassembly. Antagonist-treated cells, although their shape was altered, still had fragments of MT scattered in the cytoplasm, and most were frequently found close to their centrosome.

Microtubular components are known to be sensitive to Ca^{2+} -dependent proteolysis. Therefore, the effects of

Figure 19. Immunofluorescence staining of tubulin in 0.1 mM Ca^{2+} exposed cells in the presence of CaM antagonists.

Interphase 3T3 cells were processed according to protocol IV and exposed to 0.1 mM Ca^{2+} in the presence of 10 μM W-7 (a, b) or 20 μM TFP (c, d). Tubulin staining is shown in a and c, and DNA staining in b and d. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .



protease inhibitors on the calcium-induced disassembly was examined. 10 μ M leupeptin prevented the 0.1 mM Ca^{2+} -induced disassembly of MT (Fig. 20a, b). Cells incubated with leupeptin during the 0.1 mM Ca^{2+} exposure had a normal microtubular system. 10 μ M doses of PMSF (Fig. 20c, d) or 50 μ g/ml of soybean trypsin inhibitor (Fig. 20e, f) did not prevent the 0.1 mM Ca^{2+} -induced disassembly of MT. Cells treated with PMSF or soybean trypsin inhibitor had scattered fragments of MT in the cytoplasm with no particular organization.

Table IV summarizes the effects of different concentrations of leupeptin, PMSF, W-7 and TFP used in the 0.1 mM Ca^{2+} -induced disassembly of MT. A concentration of 2 μ M leupeptin was enough to prevent 10^{-4} M calcium-induced disassembly of MT. However, 10 μ M concentrations of either PMSF, W-7 or TFP did not prevent the calcium-induced disassembly of MT. 20 μ M of PMSF or W-7, but not W-5, did partially prevent the disassembly of MT. 0.1 mM PMSF totally prevented the 10^{-4} M calcium-induced disassembly.

The effects of 10 μ M leupeptin on the calcium-induced disassembly of MT in 48h stimulated lymphocytes were also examined. 0.1 mM Ca^{2+} disassembled most interphase MT, leaving only those close to the centrosome (Fig. 21a, b). 10 μ M leupeptin prevented this calcium-induced disassembly of MT, and a set of MT radiating from the centrosome was easily detected (Fig. 21c, d).

Figure 20. Immunofluorescence staining of tubulin in 3T3 cells exposed to 0.1 mM Ca^{2+} in the presence of protease inhibitors.

Brij-58 permeabilized cells were processed according to protocol IV and exposed to 0.1 mM Ca^{2+} in the presence of 10 μM leupeptin (a, b); 10 μM PMSF (c, d); and 50 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor (e, f). Tubulin staining is shown in a, c and e; and DNA staining in b, d and f. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .

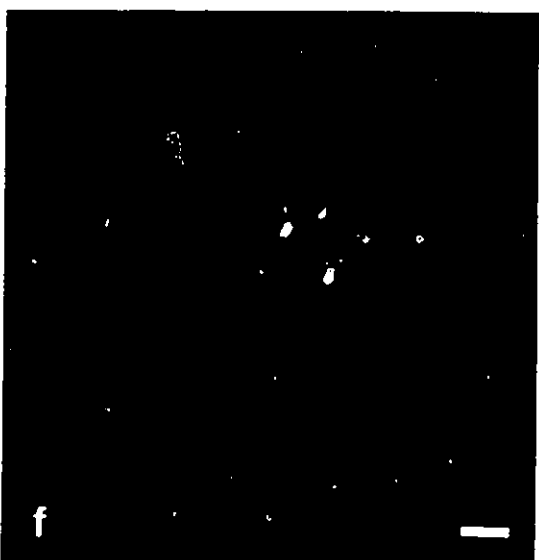
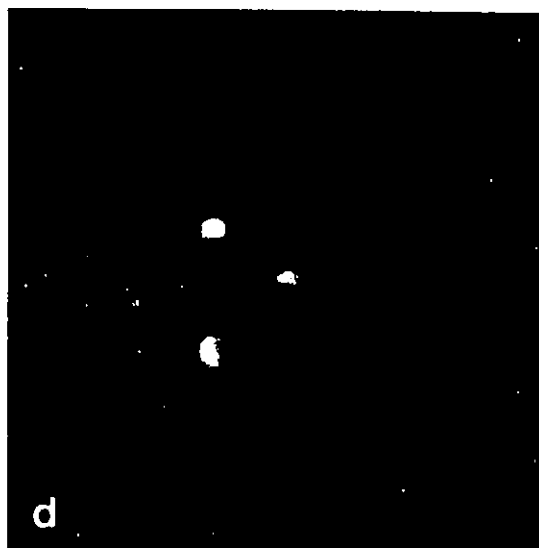
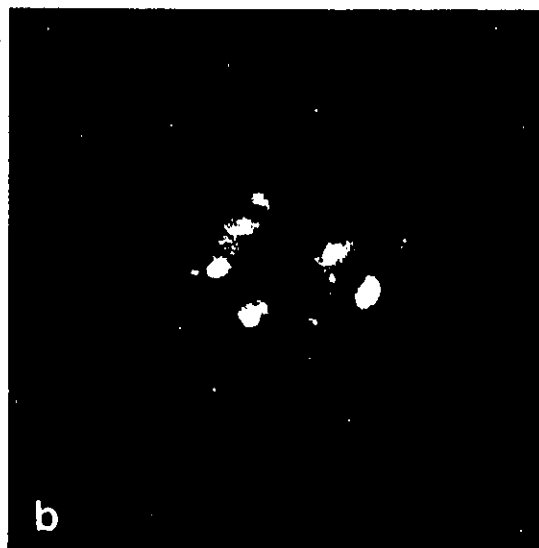
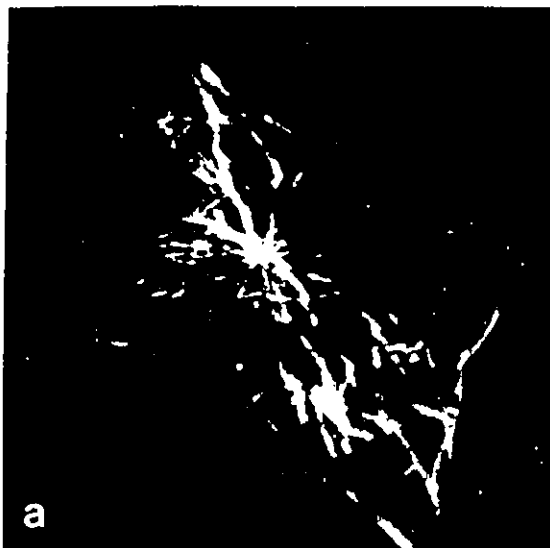


TABLE IV. Effect of protease inhibitors and CaM antagonists on the 0.1 mM CaCl_2 -induced disassembly of MT in 3T3 cells.

DRUG CONCENTRATION		% CELLS WITH NORMAL MT
Leupeptin	(10 μM)	92.2
Leupeptin	(2.0 μM)	95.2
Leupeptin	(1.0 μM)	32.0
PMSF	(0.1 mM)	100.0
PMSF	(20 μM)	37.5
PMSF	(10 μM)	20.7
PMSF	(1.0 μM)	14.7
W-7	(20 μM)	40.5
W-7	(10 μM)	23.6
W-7	(1.0 μM)	14.7
W-5	(20 μM)	0.0
TFP	(20 μM)	0.0
TFP	(10 μM)	0.0
TFP	(1.0 μM)	0.0
No Drug		0.0

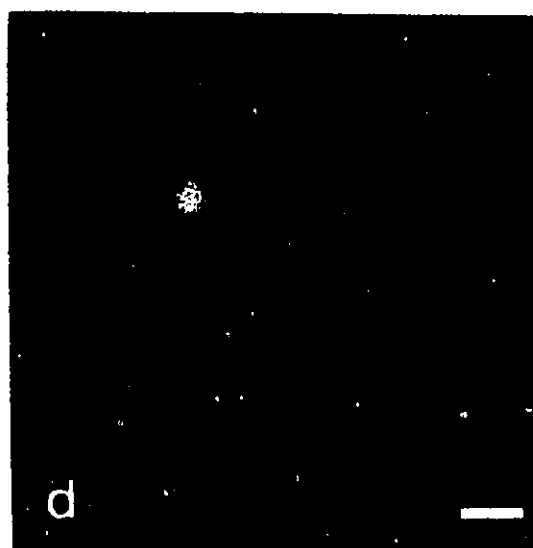
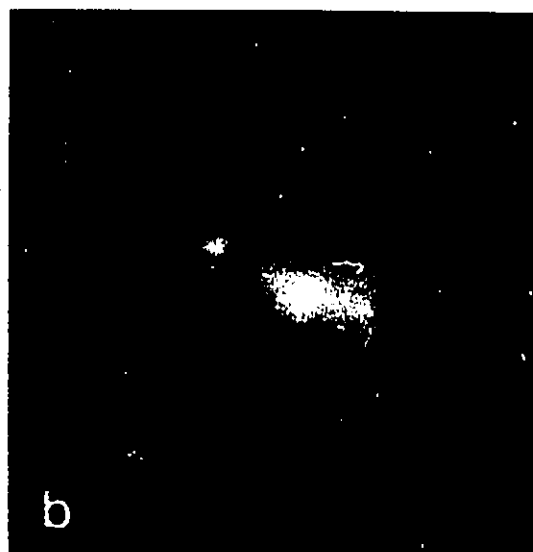
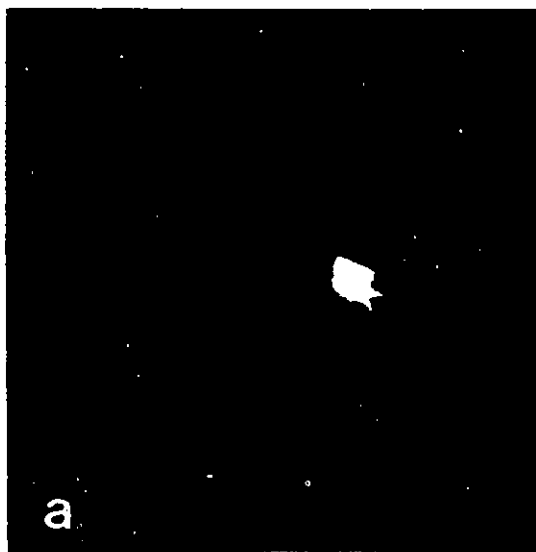
3T3 cells attached to coverslips were processed according to protocol IV and exposed to 0.1 mM Ca^{2+} in the presence of different concentrations of protease inhibitors or CaM

TABLE IV. (continued) Effect of protease inhibitors and CaM antagonists on the 0.1 mM CaCl_2 -induced disassembly of MT in 3T3 cells.

antagonists. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Five hundred cells of each drug concentration were examined. This table represents the mean of three different experiments.

Figure 21. Immunofluorescence staining of tubulin in 48h stimulated lymphocytes exposed to 0.1 mM Ca^{2+} in the presence of leupeptin.

Brij-58 permeabilized 48h lymphocytes were processed according to protocol IV and exposed to 0.1 mM Ca^{2+} in the absence (a, b) or the presence (c, d) of 10 μM leupeptin. Tubulin staining is shown in a and c, and DNA staining in b and d. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .



Since both 0.1 mM and 1.0 mM Ca^{2+} induced the disassembly of MT, the effects of 10 μM leupeptin on the 1.0 mM calcium-induced disassembly of MT were examined on 3T3 cells. Again, 10 μM leupeptin prevented the 0.1 mM Ca^{2+} -induced disassembly of MT (Fig. 22a, b). However, the same dose of leupeptin did not block the 1.0 mM Ca^{2+} -induced disassembly of MT (Fig. 22c, d).

The effect of 0.1 mM calcium on cellular tubulin

The effect of 0.1 mM calcium on MT appeared to be mediated by a proteolytic step since it was found to be irreversible and could be prevented by protease inhibitors. Therefore, the next step was to examine if tubulin was the target for the Ca^{2+} -dependent protease. First, 3T3 cells were treated with 10 μM taxol for one hour, and then the cells were permeabilized and exposed to 1 μM or 0.1 mM Ca^{2+} . Taxol-treated cells exposed to 1 μM calcium had MT arranged in bundles (Fig. 23a, b). Taxol cells treated and exposed to 0.1 mM calcium still had bundles of MT in their cytoplasm (Fig. 23c, d). Therefore, the Calpain, which is being activated upon the exposure of cells to 0.1 mM Ca^{2+} , does not act on the tubulin of the taxol-induced bundles of MT.

The second approach was to examine the effects of 0.1 mM Ca^{2+} on the proteolysis of tubulin by immunoblotting. 3T3 cells in culture dishes were permeabilized and exposed to

Figure 22. Immunofluorescence staining of tubulin in 3T3 cells exposed to 0.1 mM Ca^{2+} or 1.0 mM Ca^{2+} in the presence of leupeptin.

Brij-58 permeabilized interphase 3T3 cells were processed according to protocol IV and exposed to 0.1 mM Ca^{2+} (a, b) or 1.0 mM Ca^{2+} , both of which were in the presence of 10 μM leupeptin. Tubulin staining is shown in a and c, and DNA staining in b and d. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .

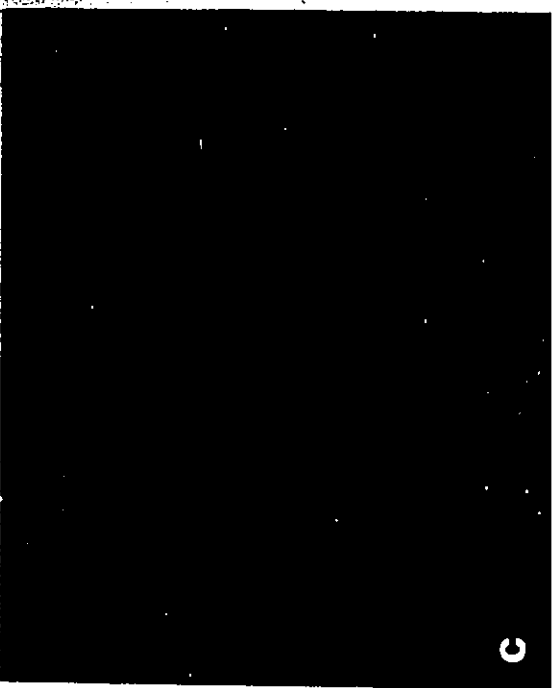
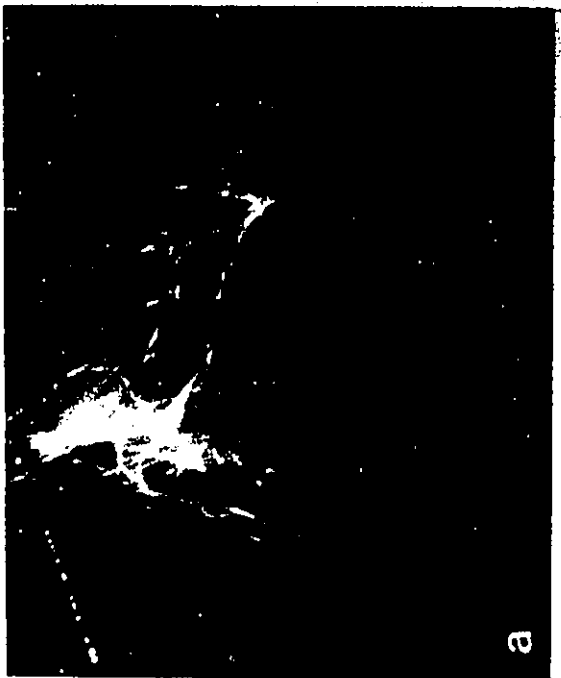
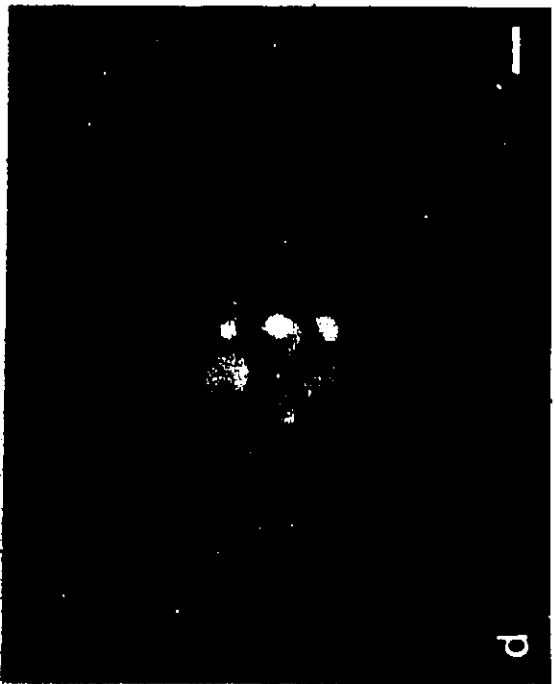
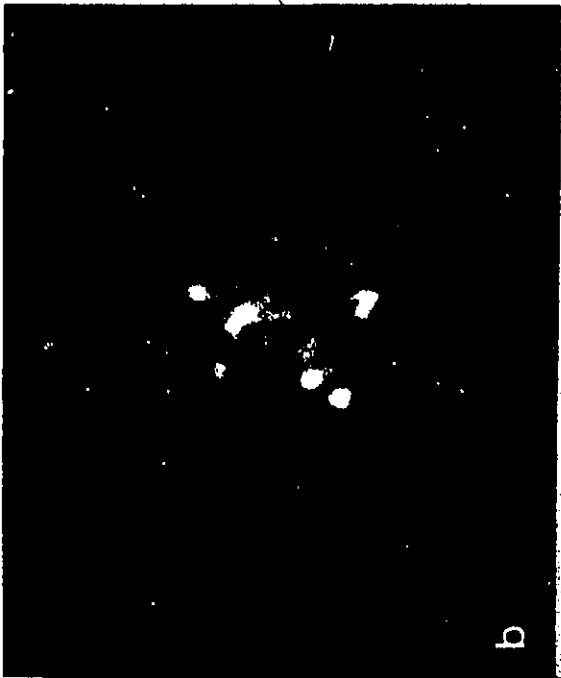
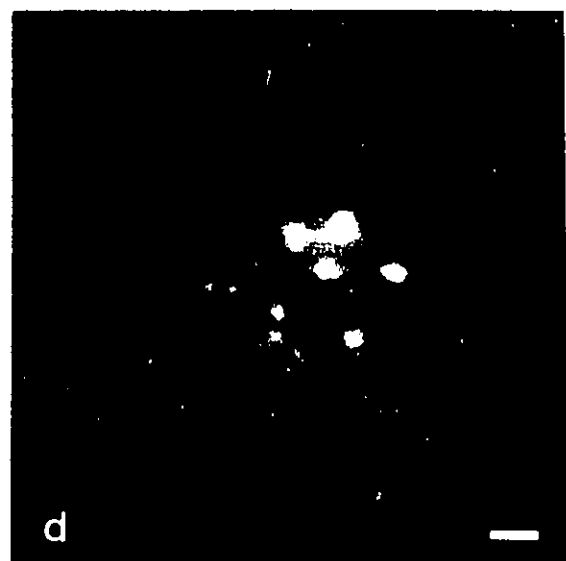
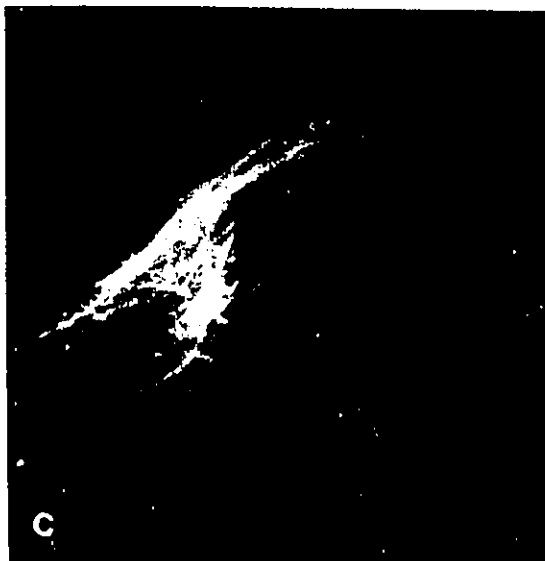
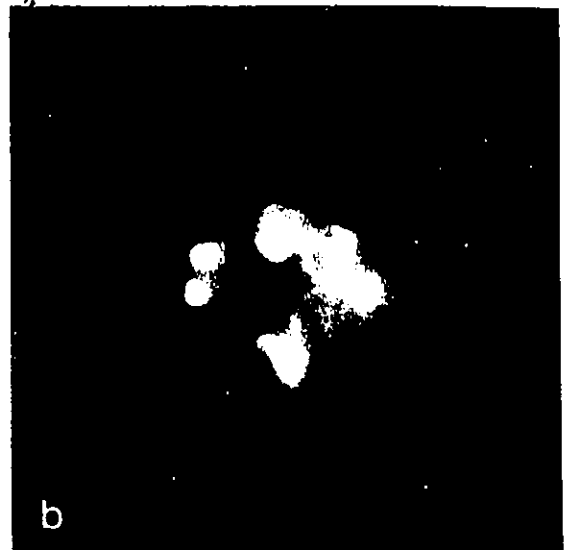
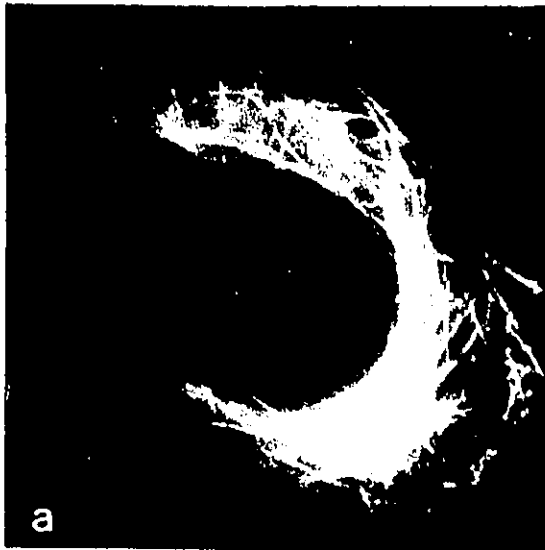


Figure 23. Immunofluorescence staining of tubulin of taxol-treated 3T3 cells exposed to 1.0 μM Ca^{2+} or 0.1 mM Ca^{2+} .

3T3 cells treated with 1.0 μM taxol for 1 hour were processed according to protocol IV and exposed to 1.0 μM Ca^{2+} (a, b) or 0.1 mM Ca^{2+} (c, d). Tubulin staining is shown in a and c, and DNA staining in b and d. Anti-tubulin was detected with an FITC-labelled anti-mouse IgG. Bar = 1.0 μm .

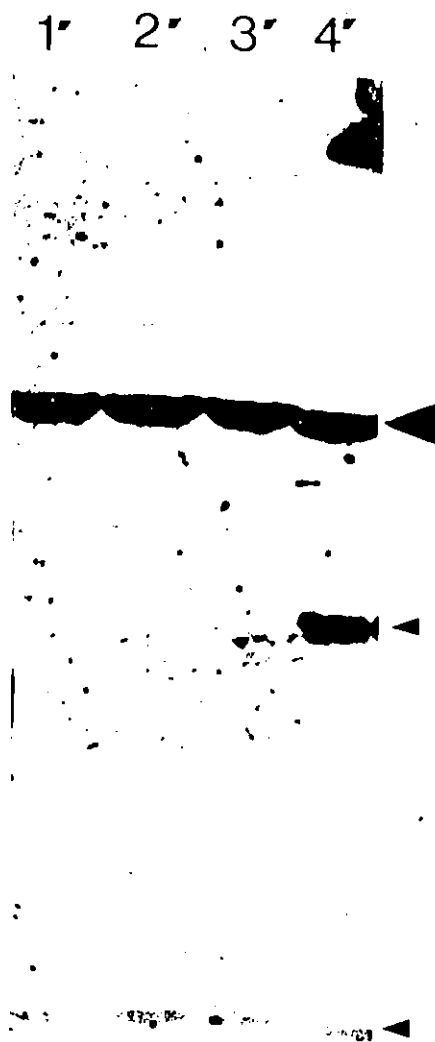


either 1 μM Ca^{2+} , 0.1 mM Ca^{2+} or 0.1 mM Ca^{2+} + 10 μM leupeptin for 10 min. The cells were scraped from the culture dish with the electrophoresis sample buffer (10 mM EGTA), homogenized and then electrophoresed, transferred to nitrocellulose and probed with the anti-tubulin 1D9.

The transfer of proteins was efficient at all the ranges of Mr (Fig. 24, lanes s, and 1 to 4). The immunoblot showed that anti-tubulin recognized two proteins of Mr 53 kD and 13.1 kD, respectively, in cells treated with 0.1 mM Ca^{2+} + 10 μM leupeptin (Fig. 24, lane 1'); 0.1 mM Ca^{2+} and no leupeptin (Fig. 24, lane 2') and 1 μM Ca^{2+} (Fig. 24, lane 3'). Non-permeabilized cells, in addition to the two components of Mr 53 kD and 13.1 kD, had a third component of Mr 33.3 kD (Fig. 24, lane 4). Therefore, it is evident that no further proteolysis occurs in cells treated with 0.1 mM Ca^{2+} versus cells treated with 0.1 mM Ca^{2+} + leupeptin or 1.0 μM Ca^{2+} . This is suggesting that tubulin is not the target for the Calpain activated upon the addition of 0.1 mM Ca^{2+} . The 33.3 kD protein detected in total 3T3 cell homogenates might be a proteolytic fragment of tubulin that is lost in premeabilized cells.

Figure 24. Immunoblotting of anti-tubulin against total cell homogenates of 3T3 cells.

Non-permeabilized cells (lanes 4, 4') or Brij-58 permeabilized 3T3 cells were exposed to 0.1 mM Ca^{2+} + 10 uM leupeptin (lanes 1, 1'); 0.1 mM Ca^{2+} (lanes 2, 2') or 1.0 uM Ca^{2+} (lanes 3, 3'). Amido black staining is shown in lanes S, 1, 2, 3 and 4), and tubulin blot in 1', 2' 3' and 4'.



DISCUSSION

In the introductory section I emphasized some of the controversial aspects, which needed more experimental evidence to further clarify the role of CaM as a mediator in the assembly/disassembly of MT. I proposed that changes in the codistribution of CaM and MT in systems other than mitotic cells might give an indication of the possible function of CaM in MT dynamics. This approach, plus the study of the effects of antimicrotubular drugs, CaM antagonists, and calcium on the assembly and disassembly of MT, might provide a more coherent picture of the calcium-CaM-MT connection.

In the first part of my research I examined the localization of CaM in the mouse T lymphocyte and its correlation with the changes of the microtubular system that occurs during stimulation by Con A. Secondly, I examined the effects of colchicine, colcemid, taxol and cold on the distribution of CaM. Thirdly, I examined the effects of several CaM antagonists on assembled MT, and MT reassembled from cold. Fourthly, the calcium-induced disassembly of MT was examined in permeabilized cells exposed to different Ca^{2+} concentrations. Finally, the effects of CaM antagonists and protease inhibitors on the calcium-induced disassembly of MT were examined.

The specificity of the antibodies

Indirect immunofluorescence was used to detect the distribution of CaM and MT in the mouse T lymphocyte. Immunofluorescence has, as an advantage, the capacity to analyze individual cells, the high resolution of the microscope and the powerful specificity of the antibodies. However, immunofluorescence also has potential problems that should always be taken into consideration. The most important one is the possibility for the antibodies to cross-react with more than one antigen within the cell.

When 3T3 cells and mouse T lymphocytes were first fixed and then permeabilized, CaM was found diffusely distributed during interphase of both cell types. In mitotic 3T3 cells and mouse T lymphocytes, CaM, in addition to its diffuse pattern, was also detected in the mitotic apparatus, showing the same pattern already described by others, with a variety of procedures and in different cell types (90). Since the CaM-adsorbed antibody did not stain 3T3 cells and lymphocytes, I assumed that this particular antibody indeed detects CaM in both cell types.

An immunoblotting procedure was used to further examine the specificity of the sheep anti-CaM and 1D9. The validity of the immunoblotting procedure as a tool to evaluate the specificity of antibodies is two-fold: 1) the cellular components are separated by electrophoresis and therefore

probed individually, and 2) those components are adsorbed to a solid hydrophobic phase which "mimics" their intracellular condition. With this technique, the anti-CaM reacted with a single component from total cell homogenates of mouse T lymphocytes, heart, kidney and intestine. That particular component had a similar Mr of bovine brain CaM. 1D9 reacted only with a single component of a Mr 52.6 kD in mouse T lymphocytes and kidney. Tubulin was the most likely protein being recognized by 1D9 by the immunoblotting, since this antibody recognized cellular microtubules by a variety of protocols for immunofluorescence used during this research.

The subcellular distribution of CaM

Immunocytochemical labelling at light and electron microscopic levels has detected CaM in almost every subcellular component. That includes plasma membrane, microtubules, stress fibers, glycogen granules, cilia and flagella, basal bodies, centrosomes, mitochondria, chromatin and mitotic spindles (31, 69-71, 90-96). In addition, a diffuse staining has almost invariably been detected in interphase and mitotic cells. This quasi-ubiquitous subcellular distribution of CaM might reflect its ability to modify the activity of over thirty different cellular enzymes and processes. Furthermore, intracellular translocations have indeed been seen in several cell types upon hormonal

stimulation (49-52). Altogether, the data indicate that CaM is a highly dynamic molecule that can be distributed in most subcellular components.

The role of CaM in microtubule function is not well understood (see Introduction). CaM localization in some mitotic MT has been known for almost ten years, whereas CaM has not been consistently localized in interphase MT. For instance, CaM was found in some neural MT (94) and in cultured cells (96). However, DeMey et al. (93) failed to detect CaM in MT and the centrosome during interphase in cultured cells. Willingham et al. (95) also failed to detect CaM in association with interphase MT, but they found some CaM localized in the centrosomal region.

The inconsistencies of the CaM localization in the centrosome and MT during interphase, could be attributed to at least two different reasons. Firstly, the different fixation protocols and anti-CaM antibodies used could be responsible for discrepancies in CaM localization. Secondly, the different localizations of CaM in the microtubular system could be real and somehow reflect dynamic redistributions of CaM and the changes of reorganization and activity of the centrosome that occur during the cell cycle.

My own research in mouse T lymphocytes has shown that under specific conditions (protocol II) involving gentle permeabilization followed by fixation of the cells, CaM was not localized in any microtubular structure of resting or 24h

stimulated lymphocyte. However, CaM colocalized with the centrosome and some MT in the 48h stimulated lymphocyte and throughout mitosis. Thus, here it is clearly evident that CaM becomes associated with the centrosome when there is an increased number of MT, suggesting a role of CaM during the assembly of microtubules.

The interaction of CaM with the lymphocyte centrosome during mitosis was very similar to the one described for other animal cells (90). In a previous study, however, Bachvaroff et al. (132) reported that CaM was diffusely distributed in the stimulated porcine lymphocyte, and showed no association with the mitotic spindle. Their failure to detect CaM in the microtubular system was possibly due to differences in protocols involved. The particular protocol II used in this study was a modification of the protocol described by Deery et al. (96) that was adapted specifically for lymphocytes.

The comparison of the subcellular CaM distribution in cells first fixed and then permeabilized (Fig. 4a) with those permeabilized in Triton X-100 first and then fixed (Fig. 7m), suggests that there is some Triton X-100 soluble CaM. This "soluble" CaM might be responsible for the diffuse localization of CaM in interphase cells first fixed, therefore obscuring the microscopic visualization of CaM on the centrosome.

The remote possibility that CaM is artifactually relocalized to the centrosome or MT by the permeabilization step before fixation (protocol II) can never be ruled out. However, this seems unlikely since neither resting nor 24h stimulated lymphocytes showed any CaM colocalized with the microtubular system.

One current hypothesis suggests that CaM might interact with MT through a calcium-dependent interaction with cytochalbins (84). CaM also might affect MT by regulating the phosphorylation of MAP2 and STOP (85,86) by calcicmodulin-dependent kinases and phosphatases.

When 48h stimulated lymphocytes were processed with 1.2 mM CaCl_2 , no MT were detected, and CaM appeared diffusely distributed in the lymphocyte. Interestingly, in mitotic lymphocytes some MT were found resistant to 1.2 mM CaCl_2 . The CaCl_2 -resistant MT appeared to be in half spindle, and had CaM localized on them. When 48h stimulated lymphocytes were processed in the presence of 10 mM EGTA and no CaCl_2 , the CaM-centrosome interaction in interphase and mitotic cells remained intact. Since 10 mM EGTA in the buffers used should result in a calcium concentration below 0.01 μM , the CaM-centrosome and CaM-mitotic spindle might be calcium-independent.

Since the CaM-microtubular system interaction does not require calcium, CaM might interact with a protein other than cytochalbins. Calcium-independent interactions of CaM with

elements of the cytoplasmic matrix have been reported previously (41-43). However, the significance of calcium-independent interactions of CaM with cytoplasmic matrix is still unclear. It has been proposed that CaM associated directly with MT will "ensure the calcium sensitivity of both mitotic and cytoplasmic microtubules" (96). Alternatively, the localization of CaM on the centrosome and MT, might provide the microtubular system with a "built in" calcium chelator, thus preventing the harmful effects of the calcium ion.

The effects of colchicine, colcemid, cold and taxol on
the CaM-microtubular system interaction

The CaM-centrosome in the 48h stimulated lymphocyte was found to be colchicine and colcemid resistant and cold-sensitive. An interaction between CaM with colcemid and cold-resistant MT of 3T3 cells had been described by Deery et al. (96). In stimulated lymphocytes, CaM might directly interact with the satellite bodies of the centrosome, which had been previously reported to be resistant to colchicine and cold-sensitive (119). The precise localization of CaM in the centrosome must be determined by electron microscopic immunolabelling.

The disruption of CaM-centrosome interaction by cold was found to be reversible upon the rewarming of the cells. It was evident that CaM became associated with the centrosome

and some MT during their reassembly. Thus this is suggesting a possible role of CaM during the assembly of MT from the centrosome.

Brown et al. (152) have shown previously that taxol-treated lymphocytes form bundles of MT originating from the centrosome in interphase cells and multiple asters in mitotic cells. Taxol decreases the critical concentration required to assemble MT in vitro (153) and also promotes the lateral interactions between the MT in vivo (154), therefore increasing their stability. Calmodulin was colocalized with taxol-induced bundles of MT and with the multiple asters of mitotic cells, regardless of the time of taxol exposure. Thus, the taxol-induced reorganization of the microtubular system did not disrupt its interaction with CaM, and the CaM-microtubular system interaction was more evident in conditions that promoted the assembly of MT in stimulated lymphocytes (i.e. reassembly from cold, taxol-induced bundles of MT). Finally, the possibility of an involvement of CaM during the assembly of MT is indirectly supported by the recent findings of Fulton, Cheng, and Lai (155). They reported a coordinate and parallel expression of CaM and tubulin genes during the formation of flagella in Naegleria.

Calmodulin antagonists and the reassembly of MT from the
centrosome

Calmodulin antagonists did not have any significant effects on viability, nor did they have an effect on MT of 48h stimulated lymphocytes at 37°C. However, CaM antagonists prevented the reassembly of MT in cold-treated lymphocytes. The effect of CaM antagonists appeared to be on the CaM activity because the effective dose was within the range of their antagonistic effects in vitro (57). Further, the inactive analog W-5 did not have any significant effect on the reassembly of MT.

The exact mechanism of action of the antagonists on the reassembly of MT is not known. Since these CaM antagonists did not affect MT at 37°C, the drugs might have blocked a particular pool of CaM, which became active and was required only at the reassembly of MT. Those drugs apparently bind to the hydrophobic domain of CaM upon its binding to calcium. Thus, CaM antagonists might have blocked the association of CaM with its binding site in the centrosome that occurs during the reassembly of MT from cold. CaM might also regulate the local calcium concentration at the time of nucleation and assembly of MT. The calcium-sequestering activity by CaM has been shown to be required for the assembly of MT in vitro when no MAPs are present (83). Thus, the antagonistic effect of anti-CaM drugs on the reassembly

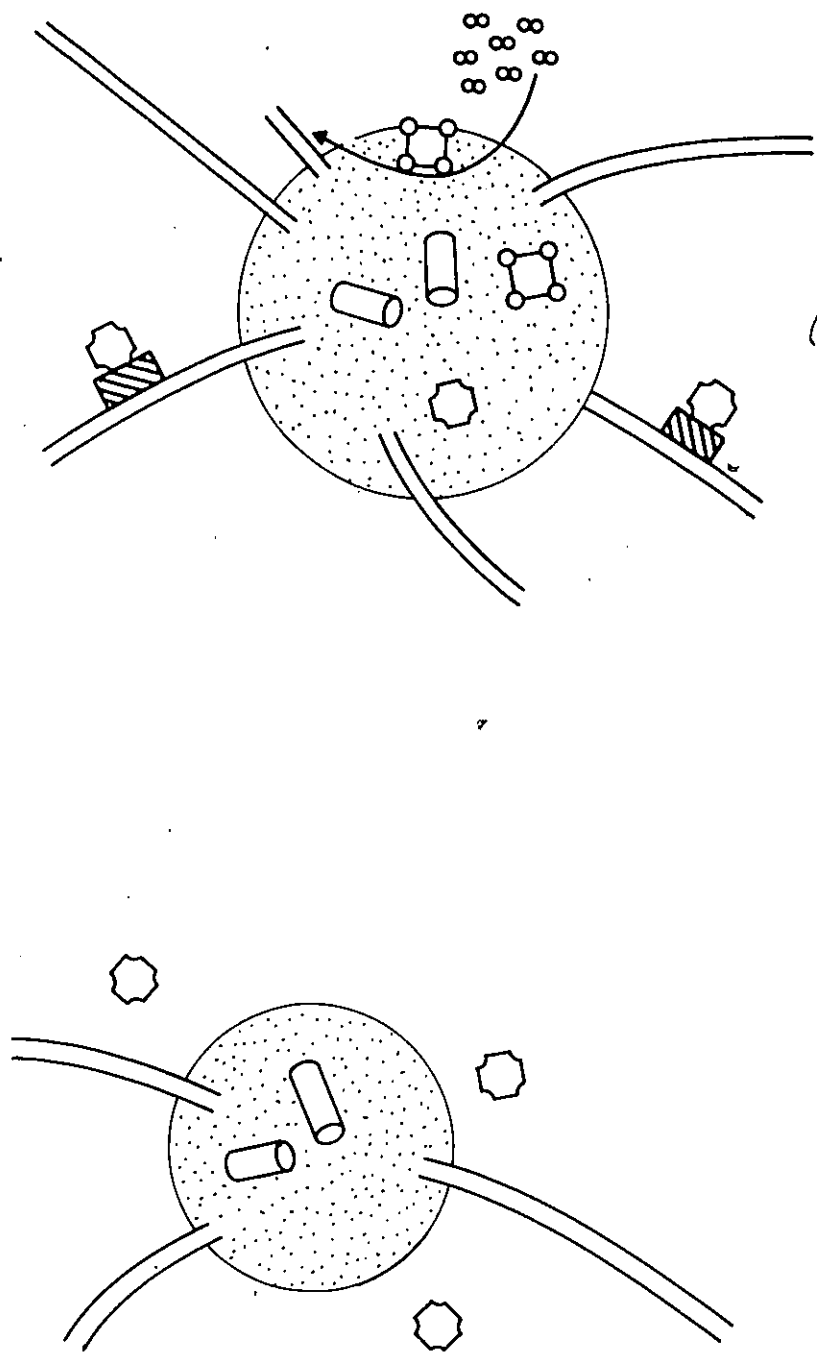
of MT might have blocked the CaM-centrosome interaction, which resulted in increased local calcium concentrations that prevented the reassembly of MT.

The distribution of CaM in mouse T lymphocytes

Figure 25 summarizes the findings on the localization and activity of CaM during the stimulation of mouse T lymphocytes. Calmodulin was not localized on the microtubular system of resting or 24h stimulated lymphocytes. However, CaM became colocalized on the centrosome and some MT when microtubular nucleating activity of the centrosome was increased in the 48h stimulated lymphocyte. The CaM-centrosome and CaM-MT interactions were found to be calcium-independent. CaM might interact with the microtubular system with a non-cytocalbin protein, since (at least in vitro) cytochalbins interact with CaM in the presence of calcium. The CaM activity is required in the centrosome since CaM antagonists have been shown to block the reassembly of MT. The proposed CaM localization and interactions shown in figure 25 do not exclude the possibility that CaM interacts with other MAP like tau, STOP and MAP2. However, the relevance of these calcium-dependent-CaM-cytochalbin interactions remains to be demonstrated within the cell. This latter point is emphasized because it should be clear

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Figure 25. The distribution and activity of CaM on the microtubular system of lymphocytes during stimulation by con A.



RESTING CENTROSOME

ACTIVE CENTROSOME

-  : Microtubules
-  8 : Tubulin dimers
-  : Calmodulin
-  O : Calcium

that the role of calcium and CaM in the assembly and disassembly of MT has not been totally elucidated.

Other CaBP, in addition to calmodulin, have been localized in centrosomes and other MTOC (156). At the present time, it is difficult to propose a unifying hypothesis for the role of CaBP in the function of the centrosome, and I can only summarize the available evidence. Calmodulin and other CaBP appear to be involved in MTOC duplication. CaM antagonists have been shown to block centrosome duplication in Hela cells (157). The yeast CDC31 mutant gene has been shown to have homology with calmodulin (158). This mutant is defective in spindle pole body (MTOC) duplication. Thus, the role of CaBP in MTOC might be multifaceted, and a better understanding of the CaBP-MTOC relationship must await more experimental evidence.

The effects of calcium on the disassembly of
microtubules

Calcium is known to induce the disassembly of MT, both in vivo and in vitro. Microtubules in vivo or those assembled from crude extracts are more sensitive to calcium than those MT assembled from tubulin + MAP (73). The interpretation has been that crude extracts and living cells have a factor that sensitizes MT to calcium. This calcium-sensitizing factor does not copurify with MAP or tubulin.

Stimulated T lymphocytes and 3T3 cells, both permeabilized with Brij-58, were used to study the effects of different calcium concentrations on the stability of MT. Brij-58 permeabilization of cells allows only ions and small molecules to exchange with the extracellular media (159, 160). Microtubules of both stimulated lymphocytes and 3T3 cells were stable in Ca^{2+} concentrations up to 50 μM . MT in Brij-58 permeabilized teleost retinal cone cells and BSC cells have been shown to be resistant to Ca^{2+} concentrations between 10 μM and 100 μM (145, 79). However, 0.1 mM and 1.0 mM Ca^{2+} disrupted MT in interphase cells, and the only tubulin staining that remained was in the centrosomal region in mouse T lymphocytes. Interphase 3T3 cells exposed to 0.1 mM and 1.0 mM Ca^{2+} had only scattered fragments of MT in their cytoplasm, particularly those MT closely associated with the centrosome. Mitotic cells of both types had a fraction of MT that was calcium-resistant. Those calcium-insensitive MT were found at the mitotic poles in prophase, kinetochore MT and at both sides of the midbody. Previously, it was shown that MT close to the centrosome in leukocytes (161) and 3T3 cells (159) and kinetochore MT in mitotic spindles of sea urchins (162) and BSC cells (163) were the most resistant to calcium.

What makes these particular MT more resistant to calcium is open to speculation. The calcium insensitivity could very well be due to the presence of a particular set of MAP or

tubulin. For example, post-translational modifications of MAP or tubulin may protect those proteins against the deleterious actions of calcium.

The fact that only calcium-free concentrations above 50 μM could induce disassembly of MT apparently does not support a physiological role for calcium in the mentioned process (because mammalian cells do not increase their Ca^{2+} above the micromolar range). However, local increases in Ca^{2+} concentrations might be responsible for a very selective disassembly of MT. Thus, the temporal and spatial redistribution of Ca^{2+} might modulate the calcium sensitivity of MT.

CaM has been proposed previously to act as a calcium-sensitizing factor in the disassembly of MT (92). However, there are at least three observations that argue against a role for CaM in MT disassembly. 1) The evidence obtained from in vitro assays always required a high ratio of CaM/tubulin to disassemble MT, and other CaBP were even more potent than CaM (77). 2) Only the microinjection of high concentrations of calcium-CaM (200 μM CaCl_2 in excess) into living cells disassembled MT; this disassembly was not rapidly reversible (98). 3) The microinjection of CaM into living mitotic cells did not affect the spindle structure or the rate of chromosome movement during mitosis (97).

My results indicate that a concentration of 50 μM Ca^{2+} does not disassemble MT, although this Ca^{2+} concentration

should be sufficient to form calcicmodulin within the cell (164). W-7 and TFP do not block the 0.1 mM calcium-induced disassembly of MT, suggesting that CaM might not be involved in this process. This is consistent with similar observations made by Perry et al. (165), Deery et al. (96) and Dolan, Reid and Voorheis (166), in which they did not observe any antagonistic effect of W-7 or TFP in the calcium-induced disassembly of MT. On the other hand, Schliwa et al. (79) reported a protective effect of several CaM antagonists in the calcium-induced disassembly of MT in BSC cells. The reason for the discrepancy of the results presented by Schliwa et al. is unknown.

Neither 10 μ M of PMSF nor 50 μ g/ml of STI prevented the calcium-induced disassembly of MT. However, 10 μ M of the calcium-dependent protease inhibitor leupeptin did block the 0.1 mM calcium-induced disassembly of MT in stimulated lymphocytes and 3T3 cells. Low concentrations of leupeptin or high concentrations of PMSF (serine protease inhibitor) blocked the calcium-induced disassembly of MT, suggesting a proteolytic event upon calcium exposure. This was further supported because the calcium-induced disassembly of MT was irreversible. To the best of my knowledge, this is the first evidence of a possible role for Calpains in the disassembly of MT within the cell.

Since leupeptin did not block the 1.0 mM calcium-induced disassembly of MT, this suggested two mechanisms of calcium

action: 0.10 mM Ca^{2+} would only be sufficient to activate Calpains, and the deleterious effects of calcium would depend totally on the activity and lifetime of those enzymes; however, 1.0 mM Ca^{2+} would provide a high excess of Ca^{2+} within the cell, which then might be bound by the calcium-binding sites of tubulin (167), thus preventing the reassembly of MT.

The 0.1 mM Ca^{2+} exposure to permeabilized 3T3 cells did not affect MT stabilized by taxol, nor did it enhance the proteolysis of tubulin. Therefore, 0.1 mM calcium might affect protein(s) other than tubulin.

MAP2 and other cytoskeletal proteins are known to be extremely sensitive to calcium-dependent proteolysis in vitro (18, 102, 103). The calcium-dependent proteolysis of MAP2 abolished its capacity to promote the assembly of MT in vitro (18). Therefore, the most likely protein(s) being affected during the 0.10 mM calcium-induced disassembly of MT in cultured cells may be similar to MAPs. This is further supported because the reintroduction of MAPs into Triton X-100-permeabilized BSC cells prevented the calcium-induced disassembly of MT (79).

Since the calcium-induced disassembly of MT appears to require a proteolytic event, the reassembly of MT should wait until the cellular levels of the proteolyzed protein are restored. This may explain why the full reassembly of MT after a calcium-induced disassembly requires about two hours

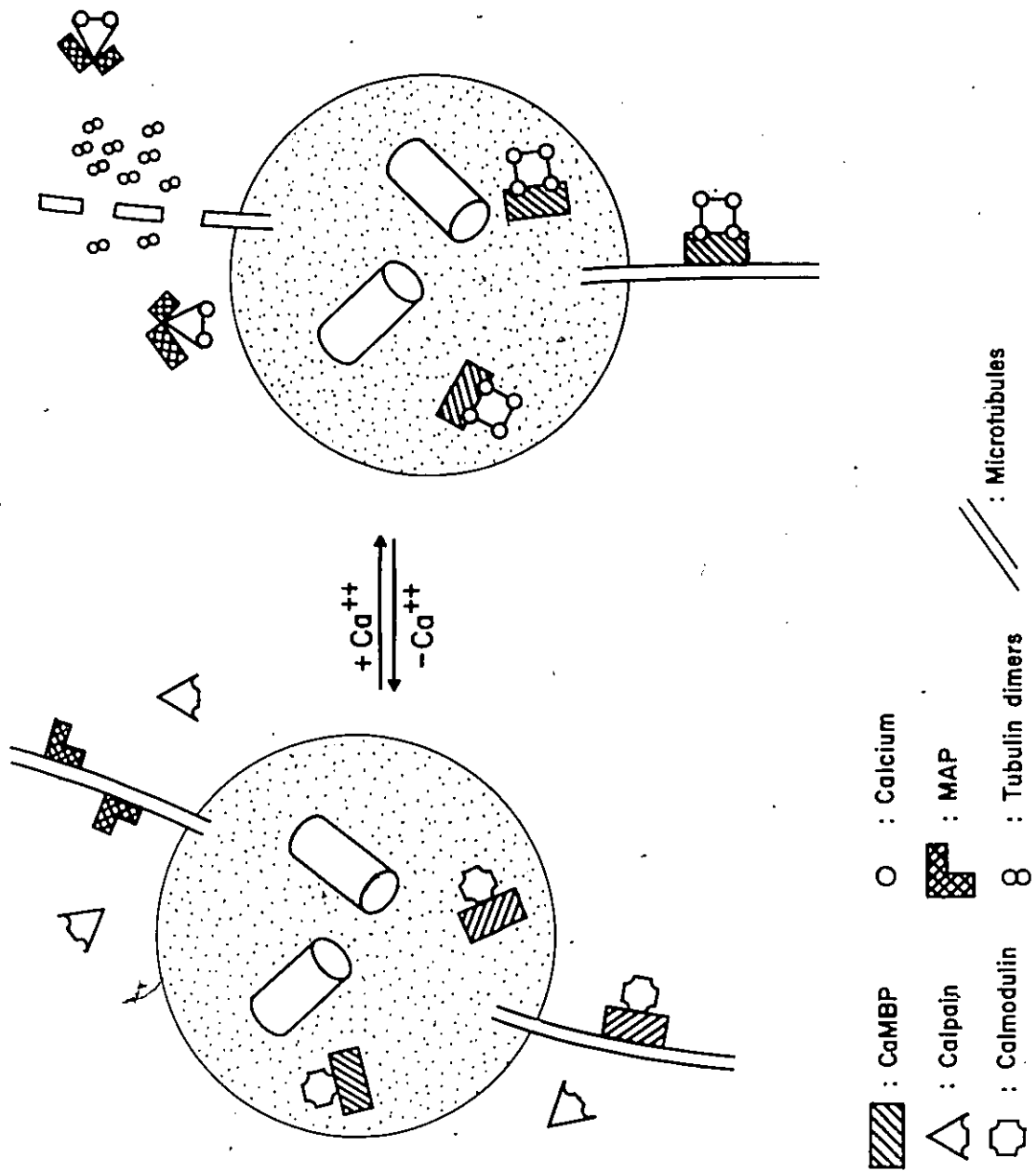
in the axopodium of Heliozoans (76) and more than 75 minutes in gerbil glioma cells (98). The high capacity of sea urchin eggs to reassemble MT after a 1 mM calcium microinjection (22) might be due to the rapid sequestration of calcium and the high content of non-assembled MAPs and tubulin already available in the eggs (168).

Figure 26 illustrates a proposed model for the calcium-induced assembly of MT. It is suggested that exposure of cells to 0.10 mM Ca^{2+} would activate a Calpain, which subsequently cleaves a "MAP", thus promoting the disassembly of MT. The "MAP" would be associated only with the calcium-sensitive MT. Interestingly, CaM is already known to be associated with the calcium-resistant fraction of MT through its interaction with an unknown MAP in a calcium-independent manner.

Conclusions and future studies

Monospecific antibodies to CaM and tubulin were used in immunofluorescence to study the relationship between the subcellular localization of CaM during the mitogenic stimulation of mouse T lymphocytes. The localization of CaM in the centrosome and some MT in lymphocytes, always corresponded to the extensive microtubular system found in the fully stimulated cell and in conditions which promoted

Figure 26. The proposed mechanism during the calcium-induced disassembly of microtubules.



the assembly of MT from the centrosome. The CaM-microtubular system interaction remained throughout mitosis.

The localization of CaM on centrosomes and the mitotic apparatus was found to be Ca^{2+} -independent, suggesting that CaM might interact with an unknown microtubular component. Furthermore, CaM was localized with the calcium-resistant MT in mitotic cells. Thus, this is providing direct evidence that a particular localization of CaM on MT is not an indicator of MT disassembly.

The CaM-centrosome interaction was found colchicine and colcemid resistant, but cold-sensitive, therefore suggesting that CaM might interact with the satellite bodies in the centrosome, since those structures also have the same characteristics mentioned above.

The specific localization of CaM in the centrosomal region will require immunolabelling at the electron microscopic level. With this structural approach, it may be possible to estimate the CaM density in the MT nucleating sites, as well as to visualize the fate of CaM when those nucleating sites are disrupted, such as after cold treatment.

Calmodulin activity appears to be required for MT reassembly after cold treatment. Thus, through the interference with CaM activity on the centrosome, it may be possible to modulate the MT assembly capacity of the centrosome. For example, the microinjection of anti-CaM antibodies, MAPs, CaMBP, CaM antagonists, Ca^{2+} , EGTA, etc.

shortly before the reassembly of MT might alter the activity of CaM and centrosome. This experimental approach could give more information about the role of CaM in the microtubular system.

In the long term, complete identification of the CaMBP in the centrosome should be possible. This will require the isolation of centrosomes from 48h stimulated T lymphocytes, followed by an analysis of the CaMBP present in the centrosomal preparation.

The study of the calcium effects indicated the presence of a calcium-resistant population of MT that closely corresponded to those MT localized near the centrosome in interphase cells, and MT in the mitotic poles, half spindles and interzone MT in mitotic cells. That particular population of calcium-resistant MT closely resembles the already known distribution of CaM in mouse T lymphocytes and 3T3 cells.

CaM did not affect the calcium-induced disassembly of MT in permeabilized cells, denying a role for CaM in the disassembly of MT. However, during these studies it has been found that the calcium-induced disassembly of MT might be mediated by a Calpain. Since tubulin itself was unaffected by the calcium-dependent proteolysis, it was suggested that the disassembly of MT might be due to the proteolysis of a "MAP". The particular distribution of this calcium-sensitive "MAP" could modulate the disassembly of MT by calcium. Thus,

the first step should be the identification of the "MAP". This might be done by the analysis of proteolytic fragments of endogenously-labelled MAPs probed by immunoprecipitation or immunoblotting. The calcium-induced proteolysis should be inhibitable by an excess of "MAP" or calpastatins introduced in permeabilized cells before the Ca^{2+} concentration is increased.

The next step could be the determination of whether Calpains have a role in the normal MT dynamics in the living cell. Limited proteolysis of microtubular components by Calpains could easily be achieved through the increase of the local calcium concentration (for instance, in the cytoplasmic face of the calcium-transport systems in plasma membrane). Thus, Calpains might be required for the regulation of the local disassembly of MT close to the plasma membrane and therefore influence diverse cellular functions such as secretion, receptor mobility, intracellular transport, etc. As a first step, these studies require the use of immunocytochemical techniques to determine the localization of Calpains within the cell.

A Calpain might be the missing calcium-sensitizing factor for the disassembly of MT reported by Nishida and Sakai (73). Finally, it appears that the calcium involvement in the dynamics of MT might be mediated by multiple factors which include CaM, MAP and Calpains. The orchestrated activity of all of those factors would then be used as

modulators to sensitize the microtubular system to the regulatory properties of calcium.

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