Disruption of spindle microtubules in living cells after microinjection of a monoclonal antibody against a 46 kDa protein from the mitotic calcium transport system

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A monoclonal antibody specific for a 46 kDa protein (anti-46 kDa) of the mitotic calcium transport system has been microinjected into kidney epithelial cells from rat kangaroo (PtK_2). The effect of the injection on the integrity of spindle and interphase microtubules has been observed using immunocytochemical techniques. Microinjection of anti-46 kDa into mitotic PtK_2 cells causes the spindle microtubules to depolymerize within a few minutes. Injection into interphase cells does not show such an immediate effect on cytoplasmic microtubules. These results stress the essential role of calcium ions in maintaining the dynamic equilibrium of mitotic microtubules with their subunits whereas interphase microtubules appear to react much slower to an interference with the intracellular calcium transport system. The result further indicates that an intact calcium transport system, regulating the calcium concentration within the mitotic apparatus, is functionally involved and essential for the progression of mitosis.

Key words: *Calcium transport, microinjection, mitotic microtubules, monoclonal antibody.*

INTRODUCTION

The regulation of mitosis is one of the fundamental questions of cell biology. The transient structure of the mitotic spindle constitute the machinery for equal distribution of the genetic material into each daughter cell. Hence, there are several competing models for understanding spindle structure and mechanics (for reviews see: Petzelt and Hafner, 1989; Whitaker and Patel, 1990; McIntosh and Pfarr, 1991; Gorbsky, 1992). Virtually all of them would agree that microtubule polymerisation and depolymerisation are necessary for mitosis. Calcium is known to effect the polymerisation of microtubules and has also been involved as a key regulatory factor in mitosis (Kiehart, 1981; Izant, 1983; Keith, 1987; Lee *et al* 1987; Tombes and Borisy, 1989). Biochemical and immunological analysis of mitotic cells have identified, among various other components, a 46 kDa protein of the mitotic calcium transport system (Petzelt and Hafner, 1986; for review see: Petzelt and Hafner, 1989; Kuriyama and Nislow, 1992). This membrane bound protein has been involved in the regulation of the calcium changes associated with the course of mitosis. A monoclonal antibody against this 46 kDa protein inhibits calcium-uptake into isolated calcium-sequestering membranes and specifically labels membranes within the mitotic apparatus of sea urchin embryos (Petzelt and Hafner, 1986). Experimentally, one way to study the problem of the involvement of a calcium transport system in mitosis within individual living cells is to use the monoclonal antibody specific for the 46 kDa protein (anti-46 kDa). The distribution

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of the calcium transport system throughout the cell can then be assessed using immunocytochemical techniques and fluorescence microscopy. After microinjection into cells one can also determine what happens not only to the progression of mitosis, but also to the spindle microtubules by using double immunolabeling with antibodies against tubulin. In sea urchin embryos, microinjection of anti-46 kDa results in an increase in the intracellular calcium concentration, a complete disappearance of the birefringence of the mitotic apparatus and an irreversible arrest of mitosis (Hafner and Petzelt, 1987). Here, we report a series of experiments in which an affinity purified antibody against the 46 kDa protein is microinjected into mitotic PtK_2 cells to further test the hypothesis that inhibition of a controlled calcium regulation at mitosis leads to a perturbation of the mitotic apparatus. In these experiments the effect of this perturbation was assessed in terms of its effect on spindle morphology, as determined by antitubulin immunofluorescent staining of injected cells. For comparison, interphase cells were also injected with the monoclonal antibody to the 46-kDa protein and their microtubule behavior investigated. These experiments extend the approach previously proven successful in sea urchin embryos (Hafner and Petzelt, 1987) and demonstrate that antibodies against components of the calcium uptake system block mitotic progression, suggesting that the regulation of calcium concentration is essential for mitosis.

MATERIAL AND METHODS

Cells and microinjection. Rat kangaroo cells (PtK_2) were grown on coverslips as described by Franke *et al* (1978): they were maintained in F-12 medium (Gibco) supplemented with 10 % fetal calf serum (Flow) and subcultured on polylysine-coated cover slips (12 mm) for 3 days before microinjection. PtK_2 cells were used to study mitotic spindles because they remain relatively flat during mitosis. Microinjection was performed as described (Ansorge, 1982) using an Eppendorf Microinjector 5242 (Eppendorf Gerätebau, Hamburg, FRG) with glass capillaries (GC150, Clark Electromedical Instruments, Reading, UK). Cover slips were mounted in 33-mm diameter observation chambers containing 3 ml culture medium. Fresh, 5% CO_2 equilibrated medium was circulated through the observation chamber at 5 ml/h throughout the experiment. The temperature was maintained at 37° C using a home-made air curtain incubator. Antibodies were used at concentrations from 2-6 mg/ml in 137 mM KCl, buffered with 10 mM sodium phosphate to pH 7.4 (reverse PBS). All solutions were stored in EGTA-washed plastic bottles. The monoclonal murine antibody specific for a 46 kDa protein prepared from a calcium uptake system of HeLa cells was described previously (Petzelt and Hafner, 1986). For microinjection of anti-46 kDa IgM, the antibody was purified from ascites fluid by ammonium sulfate precipitation, followed by affinity chromatography using anti mouse IgM-agarose (Sigma).

Immunofluorescence Microscopy. Microinjected cells were fixed after different incubation times in methanol (–20° C, 10 min). Distribution of the injected antibody was visualized by incubating coverslips with secondary antibodies conjugated to fluorescein isothiocyanate (Dianova, Hamburg) for 20 min. After intensive washing in PBS, double-labeled immunolocalisation was performed with a sheep polyclonal antibody against tubulin and subsequent incubation with secondary antibodies conjugated to Texas red (Dianova, Hamburg). For visualization of nuclei and chromosomes, cells were stained with diamidinophenylindole (DAPI). Following several washes in PBS, the coverslip forming the bottom of the incubation chamber was removed and mounted in 25 mg/ml 1,4 diazabicyclo-(2,2,2)-octane. Photographs were taken with a Zeiss photomicroscope III equipped with epifluorescence optics.

RESULTS

As seen by indirect immunofluorescence (Figs 1, 2, 3), the injected anti-46 kDa

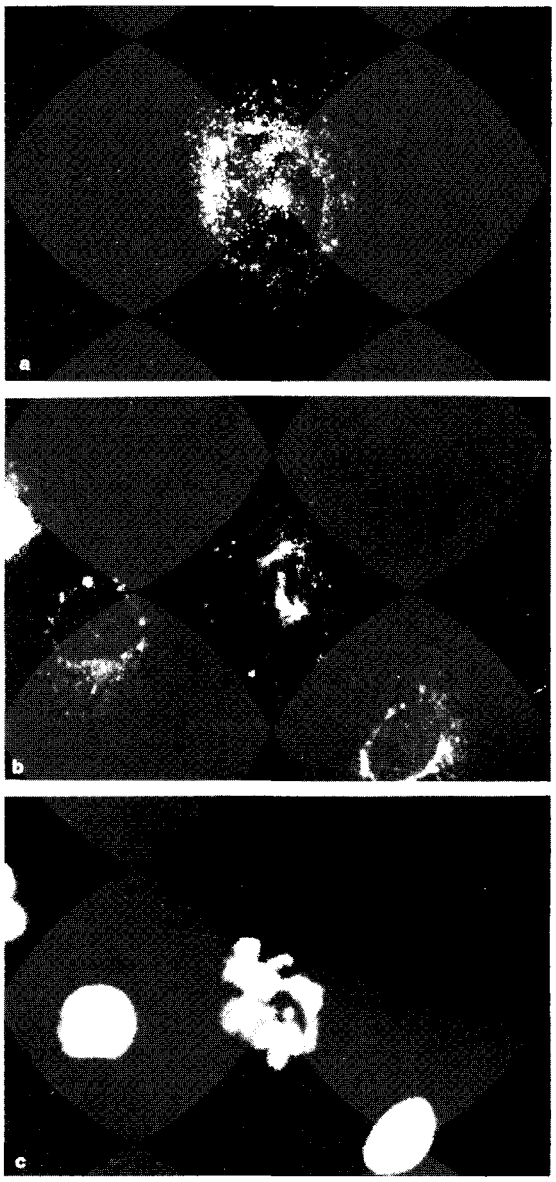

Fig 1: Beginning disintegration of spindle microtubules in PtK_2 cells injected with monoclonal anti-46 kDa. Cells fixed within 1 min after microinjection. **a**, anti-46 kDa staining (FITC). **b**, anti-tubulin staining (Texas red). **c**, DAPI staining of chromosomes.

decorated punctate patches of mitotic PtK_2 cells corresponding to the staining pattern found in mitotic sea urchin embryos (Petzelt and Hafner, 1986; Petzelt *et al*, 1987). We have previously observed (Hafner and Petzelt, 1987) that the injection of the anti-46 kDa into mitotic sea urchin embryos causes a disappearance of the birefringence of the mitotic apparatus and an arrest of mitosis. One potential mechanism by which microinjected anti-46 kDa antibody might generate mitosis inhibition would be by generating a partial or total disruption of the mitotic spindle microtubules. We, therefore, began a series of antitubulin immunofluorescence studies, in which cells undergoing mitosis were microinjected with anti-46 kDa, fixed and processed immunohistochemically for visualization of microtubules at known times after injection, to determine whether microinjected anti-46 kDa affects spindle morphology as seen at the light microscope level. Within the asynchronous cultures we used, prophase and early metaphase cells were identified in phase contrast optics. They were subsequently injected with anti-46 kDa or other control IgM antibodies. In a series of microinjection experiments, we found that the effect of the anti-46 kDa on mitotic microtubules was concentration and time dependent. Shortly after microinjection (30 sec to min), the spindle morphology was perturbed (Fig 1), showing a discontinuous staining of the kinetochore and interpolar microtubules, probably due to break in the tubulin polymers. The fragments varied in length and were spaced apart by unstained regions also variable in length. However, when fixed within 3 min after injection, severe damage to spindle microtubules was already obvious and almost no intact microtubules could be seen, whereas non-injected cells showed a normal microtubule distribution (Figs 2, 3). When the injected cells were incubated for longer time periods before fixation (> 60 min), it was evident that mitotic cells were not capable of recovering from the disintegrating effect.

In contrast to mitotic spindle microtubules, cytoplasmic microtubules were surprisingly more resistant to an anti-46 kDa injection. This is demonstrated in Figure 4: microinjected anti-46 kDa was not able to break down cytoplasmic microtubules of interphase cells, even when fixation was performed 20 min after injection.

These differences in the response of the cell at various cell cycle stages to i) the same anti-46 kDa and, ii) to other control IgM injected (not shown), allow us to conclude that we are looking at a deleterious effect

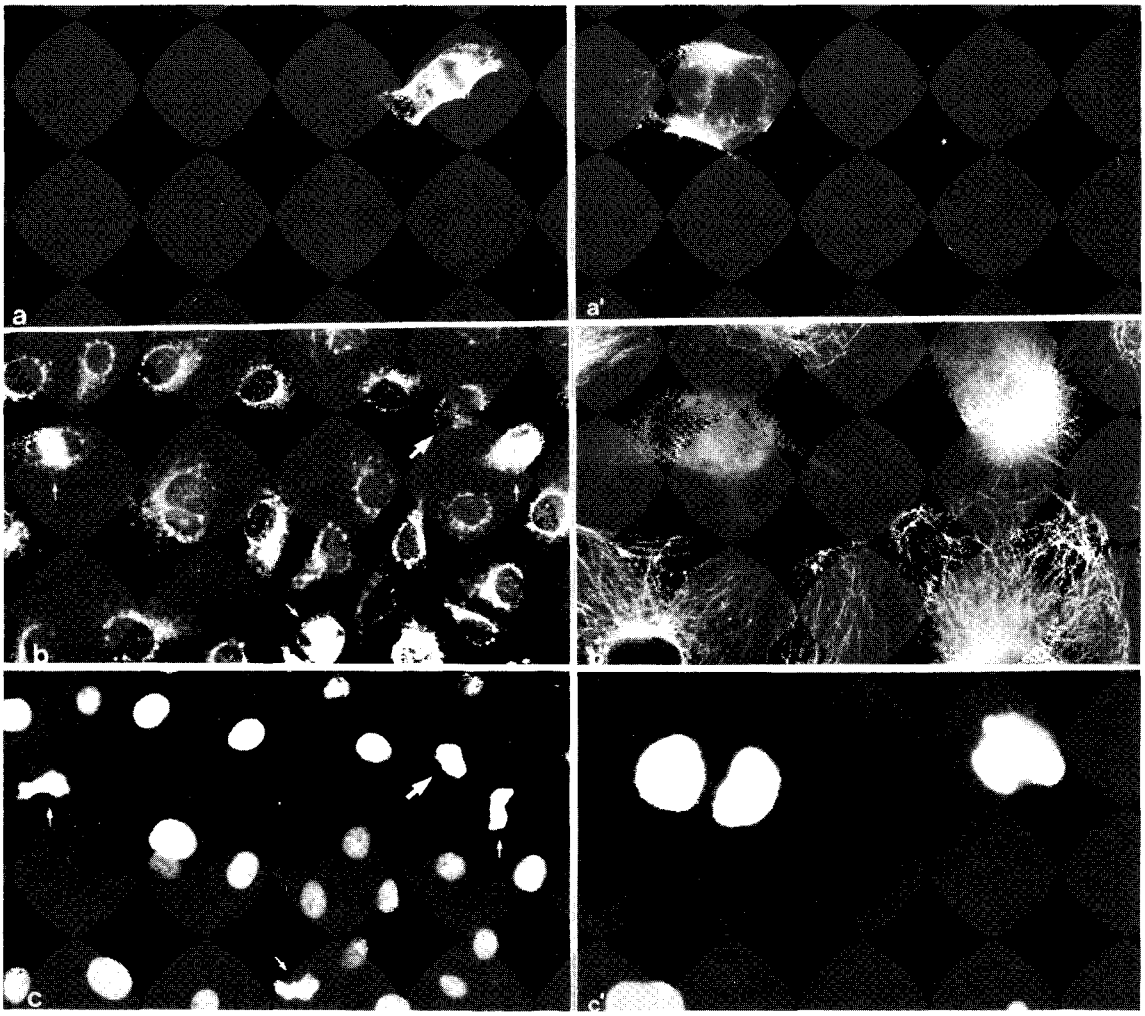

Fig 2: Complete disruption of spindle microtubules injected with anti-46 kDa. Cells fixed within 3 min after microinjection. **a**, **a'**, FITC-staining of anti-46 kDa in microinjected cells. At this time point, disintegration of spindle microtubules is almost complete. **b**, anti-tubulin staining (Texas red), non-injected mitotic cells (three little arrowheads) show intact mitotic spindles, big arrowhead points to microinjected cell with disrupted spindle microtubules. Cytoplasmic microtubules of other cells are out of the focal plane. **b'**, Details taken at higher magnification show intact spindle microtubules in non-injected cells (right). **c**, **c'**, DAPI-staining of nuclei and chromosomes.

specific to the calcium transport within the mitotic spindle and not at a transient artifact caused by the microinjection process.

DISCUSSION

Microinjection of antibodies into tissue culture cells or embryos has recently developed into an attractive method to study structure and function of complex cellular elements such as microtubules, microfilaments or membrane proteins of calcium regulation systems (Taylor and Wang, 1980; Kreis and Birchmeiert, 1982; Jockusch and Füchtbauer, 1985; Hafner and Petzelt, 1987). In those experiments specific polyclonal or monoclonal antibodies prepared against purified proteins cross-react with the corresponding antigen in embryos or tissue culture cells due to the often highly conserved nature of cytoskeletal or membrane-bound calcium control components. Introduced into the cells by microinjection and bound in antibody-antigen complexes, their effect and distribution can be followed either directly (if the antibody has been labeled with a fluorescent marker) or indirectly by labeled second antibodies after fixation of the cell.

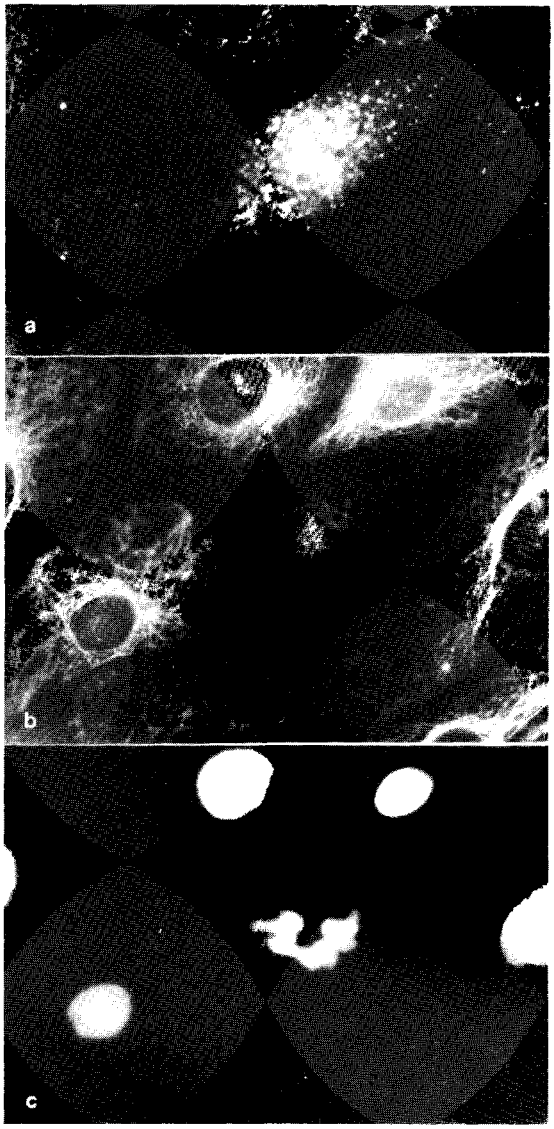

Fig 3: Induced disruption of spindle microtubules in a mitotic PtK_2 cell at higher magnification, fixed 30 min after microinjection. Cells are not able to recover. **a**, anti-46 kDa staining (FITC). **b**, anti-tubulin staining (Texas red). **c**, DAPI-staining of nuclei and chromosomes.

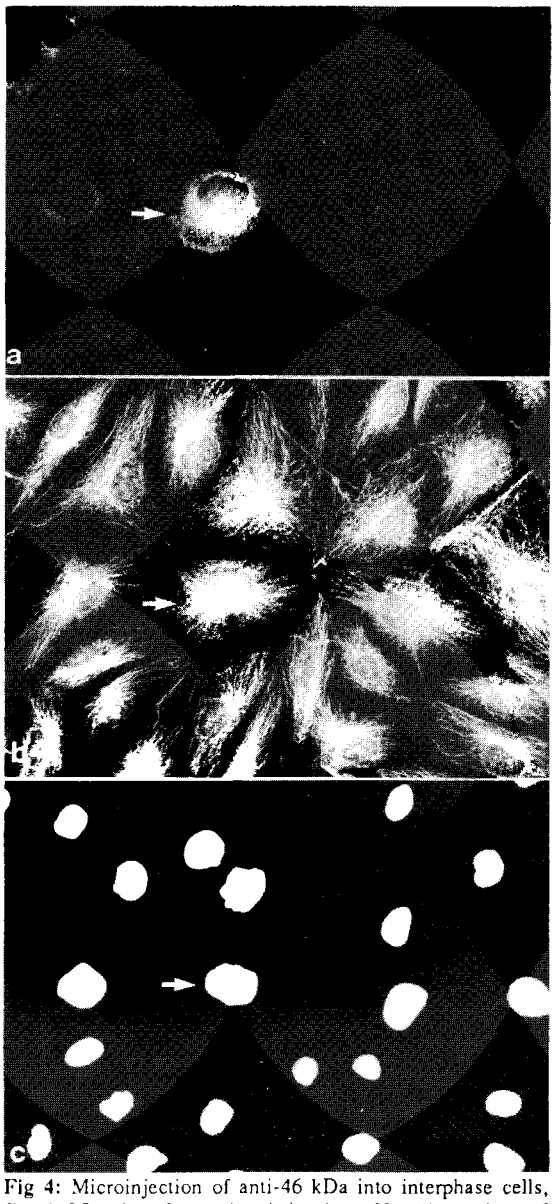

Fig 4: Microinjection of anti-46 kDa into interphase cells, fixed 25 min after microinjection. No disruption of cytoplasmic microtubules is visible. **a**, injected cell (arrow, FITC). **b**, anti-tubulin (Texas red). **c**, DAPI-staining of nuclei.

In this study we have undertaken a series of microinjection experiments in which a monoclonal antibody against a 46 kDa-protein of the mitotic calcium transport system is microinjected into mitotic PtK_2 cells to test the hypothesis that a tightly controlled calcium regulating system is essential for the structural integrity of the mitotic apparatus. Many experiments have indicated that mitosis in higher eukaryotes is a calcium-controlled process and several lines of evidence suggest that free calcium ions act as a trigger for anaphase onset in plant and animal cells. Weisenberg (1972) was the first to notice, that microtubule assembly was specifically inhibited by calcium ions. This observation fueled speculations that during mitosis calcium might control anaphase microtubule depolymerization directly. The course of mitosis is perturbed by variations in extra- and intracellular free calcium concentration: microinjection of calcium/EGTA-buffers into

echinoderm embryos (Kiehart, 1981) and mammalian tissue culture cells (Izant, 1983; Keith, 1987; Tombes and Borisy, 1989) causes a rapid but transient decrease in the organization of the mitotic spindle microtubules. In addition to the cytoskeletal elements the spindle is packed with membranes which are able to sequester and release calcium (Hepler and Wolniak, 1984; Petzelt and Wülfroth, 1984; Suprynowicz and Mazia, 1985). The sequestration activities described oscillate during the cell division cycle, with a peak activity at telophase (Suprynowicz and Mazia, 1985). This fluctuating activity has also been observed for a calcium activated ATPase (Petzelt, 1979). Antibodies to a 46 kDa-component of this ATPase show labeling that accumulates around the nucleus prior to nuclear envelope breakdown and stains the spindle during mitosis (Petzelt and Hafner, 1986; Petzelt *et al*, 1987).

Furthermore, using calcium-sensitive fluorescent probes cytoplasmic calcium ion levels can be shown to vary locally during the course of mitosis (Keith *et al*, 1985; Poenie *et al*, 1985; Poenie *et al*, 1986).and meiosis (Tombes *et al*, 1992). Single abrupt rises (Poenie *et al*, 1986) as well as sustained increases (Ratan *et al*, 1986; Hepler and Callahan, 1987) have been observed at anaphase onset indicating a highly active calcium transport system. In addition to the calcium ATPase sequestration activity concentrated in the spindle, the calcium binding protein calmodulin, calpain II, a calcium activated protease and a calcium/calmodulin dependent protein kinase (CaM kinase II), are found in the mitotic apparatus (Welsh *et al*, 1979; Cheung, 1980; Zavortink *et al*, 1983; Vantard *et al*, 1985; Schollmeyer, 1988; Ohta *et al*, 1990). Furthermore, calmodulin antagonists slow the passage of cells through mitosis or even arrest cells in metaphase (Keith *et al*, 1983; Sarma and Goode, 1985). These findings involve calmodulin in the transduction of calcium ion effects on the mitotic machinery. Keith (1987) has examined the role of calmodulin in mitosis by observing the consequences of calmodulin microinjection on the progress of mitosis and morphology of the mitotic spindle in PtK_2 cells. Injection of excess calcium saturated calmodulin during early prometaphase not only significantly prolongs the time required for the cell to complete mitosis, but also has an immediate effect on spindle morphology as judged by antitubulin immunofluorescence, which closely resembled the effects described in this paper after microinjection of the anti-46 kDa. Regarding the consequences of introducing the anti-46 kDa into the cell, one can imagine the following possibility: the observed effect may indeed be mediated by microtubule-associated proteins (MAP's), tau factor, a family of closely related calmodulin-binding proteins (Kakiuchi, 1985) or CaM kinase II. MAP's appear to confer calcium stability to microtubules (Shulman *et al*, 1985) whereas tau proteins seem to act as nucleating centers for microtubule assembly. The removal of tau protein from microtubules by binding to calcium/calmodulin promotes depolymerization of the microtubules (Kakiuchi, 1985). In the mitotic apparatus of sea urchin, the calcium/calmodulin dependent phosphorylation of a 62 kDa-protein was also reported to induce depolymerization of microtubules (Dinsmore and Sloboda, 1988). The observed transient rises in the calcium concentration during metaphase may lead to the active state of these various enzymes during anaphase. The present result suggests that blocking the calcium uptake machinery by the anti-46 kDa antibody probably prevents local re-uptake of calcium leading to a deleterious continuous leakage of free calcium from membrane vesicles of the mitotic apparatus. This effect of microinjected anti-46 kDa has previously been demonstrated in sea urchin embryos, linking calcium to the integrity of spindle microtubules (Hafner and Petzelt, 1987). However, the role of calcium transients during mitosis remains still unclear, and it is rather possible that there are pathways by which calcium or calcium/calmodulin can regulate mitosis other than a mechanochemical effect on the spindle fiber. This spindle dependency has been observed with other potential target molecules that may be involved in the regulation of mitosis (for review see Whitaker and Patel, 1990; Kirschner, 1992). Here, cell cycle control

can be described by the interactions of kinases, phosphatases and phosphoproteins without the dependence on signals from cell messengers (Murray and Kirschner 1989). We do not know yet whether calcium controls or is controlled, *e. g.* by cyclin degradation or spindle protein phosphorylation. The multifaceted activity of calcium currently prevents unequivocal discrimination of primary and secondary consequences of calcium signaling, but it is less likely that entry and progression of mitosis rely upon a simple calcium signal (Tombes and Borisy, 1989). However, from what we have said so far, it emerges that a potential interaction could well be mediated through targets including calmodulin and spindle microtubules. Further experiments, *e. g.* with simultaneous subcellular localisation of free calcium (Fura-2 imaging) and calcium-activated calmodulin with new fluorescent probes (MeroCaM, Hahn *et al*, 1992), must address the complex interdependency between calcium mobilization and other mitotic regulatory components.

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This manuscript is dedicated to the memory of Prof Luis Izquierdo.

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