Studies on the In Vivo Sensitivity of Spindle Microtubules to Calcium Ions and Evidence for a Vesicular Calcium-sequestering System

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ABSTRACT  I microinjected calcium ions into echinoderm eggs during mitosis to determine the calcium sensitivity of microtubules (Mts) in vivo. Spindle birefringence (BR), a measure of the number of aligned Mts in the spindle, is locally, rapidly, and reversibly abolished by small volumes of microinjected CaCl₂ (1 mM). Rapid return of BR is followed by anaphase, and subsequent divisions are normal. Similar doses of MgCl₂, BaCl₂, KCl, NaCl, pH buffers, distilled water, or vegetable oil have no effect on spindle BR, whereas large doses of such agents sometimes cause slow, uniform loss in BR over the course of a minute or more. Of the ions tested, only Sr⁺⁺ causes effects comparable to Ca⁺⁺. Ca-EGTA buffers, containing greater than micromolar free Ca⁺⁺, abolishes BR in a manner similar to millimolar concentrations of injected CaCl₂. Caffeine, a potent uncoupler of the Ca⁺⁺-pump/ATPase of sarcoplasmic reticulum, causes a local, transient depression in spindle BR in the injected region. Finally, injection of potassium oxalate results in the formation of small, highly BR crystals, presumably Ca-oxalate, in Triton-sensitive compartments in the cytoplasm. Taken together, these findings demonstrate that spindle Mts are sensitive to levels of free Ca⁺⁺ in the physiological range, provide evidence for the existence of a strong cytoplasmic Ca⁺⁺-sequestering system, and support the notion that Mt assembly and disassembly in local regions of the spindle may be orchestrated by local changes in the cytoplasmic free Ca⁺⁺ concentration during mitosis. An appendix offers the design of a new chamber for immobilizing echinoderm eggs for injection, a new method for determining the volume of the injected solution, and a description of the microinjection technique, which was designed, but never fully described, by Hiramoto (Y. Hiramoto, Exp. Cell. Res., 1962, 27:416-426.)

Cytoplasmic microtubules (Mts) are ubiquitous cellular organelles that partake in a wide variety of cell functions, including chromosome movement, cell shape changes, intracellular transport, secretion, and changes in membrane fluidity (for review see references 53 and 67). As exemplified by the Mts that are the main structural elements of the mitotic spindle, they are labile structures that rapidly assemble and disassemble at the cell's call. This labile nature is intimately coupled to their role in cell function; spindle Mts assemble, function to coordinate chromosome movement, and then rapidly disassemble (29, 32-34). Their behavior reflects an equilibrium between Mts and their protein subunits, tubulin dimers or oligomers, that has been clearly demonstrated both in vivo (31, 57) and in vitro (4, 35, 39). It follows that subtle and local changes in the equilibrium between Mts and tubulin can affect and affect various processes in the cell.

The equilibrium assembly of Mts is, in part, controlled by Mt organizing centers that "turn on" (or "off") to promote the polymerization (or depolymerization) of Mts in a well-defined array (in vivo studies: 29, 30, 47, 65, 66; in vitro studies: 5, 24, 30, 41, 51, 63, 74). Some Mts, however, are not associated with morphologically identifiable organizing centers (14, 15, 42), suggesting that soluble factors that alter the chemical milieu of the cytoplasm may also contribute to the control of assembly.
Based on Weisenberg's (71) initial observations that calcium inhibits the assembly of Mts in crude brain extracts, calcium was among the soluble factors first put forward as possible regulators of Mt polymerization in vivo.

To confirm calcium regulation of Mt assembly in vivo, two criteria need be fulfilled; the natural assembly or disassembly of cytoplasmic Mts in vivo must be correlated with local changes in the concentration of free Ca$^{2+}$, and experimental manipulation of the free Ca$^{2+}$ concentration in cells must shift the equilibrium between Mt nucleation and their subunits. Attempts to correlate natural fluctuations in the local intracellular free Ca$^{2+}$ concentration during mitosis have to date proved unsuccessful. I microinjected calcium–containing solutions directly into dividing echinoderm eggs to transiently alter local levels of free Ca$^{2+}$. Using polarized light microscopy, I monitored the effects of injected Ca$^{2+}$ on the birefringence (BR) of the mitotic spindle, and demonstrated that spindle Mts are calcium labile in the micromolar free Ca$^{2+}$ range. Further, these experiments provide evidence for a strong Ca$^{2+}$--buffering system in sea urchin egg cytoplasm (36).

MATERIALS AND METHODS

Experimental Material

Fertilized eggs from four species of echinoderms were used: Lytechinus variegatus and Lytechinus pictus (sea urchin), Asterias forbesi (starfish), and Echinarachninus parma (sand dollar). Nearly all experiments were performed at least once on L. variegatus. Eggs from the other species were used to obtain supplemental data. No species-specific results were detected.

Collecting Gametes

Sea urchin and sand dollar eggs were collected by electroshock or KCl injection (11, 17). Starfish eggs were spawned from isolated ovaries by 1-nm-aldamine treatment (17). All eggs were washed twice with fresh, filtered seawater, and were fertilized within 2 h of spawning by adding a dilute suspension of sperm collected "dry" or by electroshock (11, 17). After 2-3 min, fertilized eggs were washed twice with fresh seawater to remove extra sperm.

Loading the Microinjection Chamber

Eggs were loaded into an injection chamber for observation before, during, and after microinjection (see Appendix). The injection chamber (a) allowed flattened eggs to develop through plutei (Lytechinus and Echinarchchnus) or dipleurula (Asterias) larvae approximately in synchrony with embryos cultured under optimal conditions in a petri dish; (b) allowed easy access to and penetration of the sea urchin egg cytoplasm (36).

Microscopy

Cells were observed and photographed with a modified Nikon Model S microscope. Illumination was by mercury arc lamp (Osrarn HBO 200; Osram Inc., Munich, W. Germany) with two heat-cut filters and a 546-nm interference filter. Plane polarized light was provided by a combination of an elliptically polarizing Glan-Thompson prism polarizer and a thin sheet of mica (retardation $\sim$15), both mounted on rotatable rings positioned below a Bress Kohler type compensator (American Optical Corp., Buffalo, N. Y., with retardation of 1/25). The use of a strain-free $\times 10$ (NA 0.25) Olympus objective as the condenser provided the long working distance needed for the thick microinjection chamber. The objectives were Nikon strain-free rectified objectives ($\times 20$, NA 0.40 and $\times 4$, NA 0.65) and an Olympus $\times 4$ (NA 0.10) strain-free objective.

Cells were photographed on 35-mm Kodak Plus X film processed in a 1:3 dilution of Microdol X at 24°C for 9.5 min.

Experimental Protocol

Details of the microinjection apparatus and general procedures for its use are given in the appendix.

In these studies, experimental solutions were directly microinjected into local regions of the egg cytoplasm during mitosis. Because the BR of the spindle is a function of the number and packing density of its constituent Mts (60), the effect of these solutions on spindle Mts in living, dividing eggs could be ascertained by evaluating changes in spindle BR. Such changes were assessed qualitatively as changes in the brightness of the spindle, as viewed on a series of photographs taken at different times before and after injection. As long as the orientation of the photographic exposure remained constant, the optical density of the spindle on the photographic negative was a monotonically increasing function of its BR (56).

CaCl$_2$ and Control Injections:

To investigate the effects of CaCl$_2$ solutions on spindle BR, I injected $>50$ echinoderm eggs during mitosis, usually between mid prometaphase and early anaphase (concentration and volume given in Table I). Unless otherwise stated, one basic protocol was used for all injections. The pipette tip was positioned at the spindle pole and the entire volume (equivalent to 0.2–10% the volume of a Lytechinus or Asterias egg) was injected in $<0.25$ s. Just before injection, I verified that the micropipette tip and the spindle were in close proximity vertically, as well as horizontally, by gently deflecting the spindle pole with the tip of the micropipette.

Table II outlines the composition and volume of the solutions I rapidly injected at the spindle pole as controls. The basic protocol of a few experiments was varied as follows. In six eggs, I slowly (over the course of 20–80 s) injected 5 mM CaCl$_2$ at the spindle pole to evaluate the effect of the rate of injection on spindle BR. I also rapidly injected CaCl$_2$ into the cytoplasm away from the spindle pole.

Ca$^{2+}$-EGTA BUFFER SOLUTIONS: A series of Ca-EGTA buffer solutions (each adjusted to pH 6.80) were rapidly injected at the spindle pole to establish the minimum concentration of free Ca$^{2+}$ required to abolish spindle BR (Table III). With the same basic protocol, I also injected a solution of 9.5 mM CaCl$_2$ and 10 mM EGTA, and in a few experiments I slowly (15–60 s) injected a solution of 95 mM CaCl$_2$ and 100 mM EGTA. Free Ca$^{2+}$ concentration was calculated for each Ca-EGTA solution using the apparent affinity constant for Ca-EGTA binding given by Allen et al. (log $K_a$ = 6.45; 1]).

CAFFEINE AND POTASSIUM OXALATE INJECTIONS: To further probe for a vesicular Ca$^{2+}$ sequestering system in the cytoplasm of these eggs, I injected caffeine or potassium oxalate (K-oxalate) into these eggs (Table IV). K-oxalate (pH = 6.80) is readily soluble in aqueous solution, but the Ca$^{2+}$ salt of oxalate is highly insoluble (K$_{sol} = 2 \times 10^{-10}$ M), and the crystalline precipitates that result are readily visible in polarized light.

RESULTS

CaCl$_2$ Injections

When rapidly injected at the mitotic spindle pole, >7 pl of 1, 3, or 5 mM CaCl$_2$ immediately (<<1 s) abolishes the BR of the spindle around the tip of the micropipette (Figs. 1 and 2). Similar doses (dose = concentration $\times$ volume) of CaCl$_2$ injected in the cytoplasm, away from the spindle have no effect on spindle BR. Concentrations of CaCl$_2$ >5–10 mM coagulate the cytoplasm and a precipitation membrane, resembling the surface precipitation membrane described by Heilbrunn (25) and Gross (18), forms. Spindle BR is not always abolished if <3–7 pl of 1 mM CaCl$_2$ is injected, and only rarely do even large volumes (>50 pl) of 0.1 mM CaCl$_2$ abolish spindle BR (Table I). Within seconds after spindle BR is abolished by CaCl$_2$ injection, BR begins to return and spindle fibers and

| Concentration (mM) | No. of cells | Volume, pl | Average (Range) | No. effect | Rapid depolymerization |
|-------------------|-------------|------------|----------------|------------|------------------------|
| 0.1               | 5           | 28         | (14–53)        | 4          | 1                      |
| 1                 | 24          | 32         | (4–93)         | 6          | 18                     |
| 3                 | 6           | 24         | (14–29)        | 1          | 5                      |
| 5                 | 22          | 21         | (6–49)         | 1          | 21                     |
| 10                | 10          | 8          | (1–20)         | 1          | 9                      |

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**TABLE III**

*Ca** -EGTA Buffer Injections

| Injected agent | No. of cells | Volume, pl (Range) | Results (no. of cells) |
|----------------|-------------|--------------------|------------------------|
| Wesson oil     | 6           | 187 (104-418)      | No effect (6)          |
| Distilled water| 11          | 53 (19-175)        | At high volumes, slow partial loss |
| 5 mM MgCl₂     | 8           | 39 (25-51)         | No effect (5)          |
| 20 mM MgCl₂    | 4           | 18 (14-29)         | Slow, partial loss of BR (3) |
| 50 mM MgCl₂    | 6           | 23 (15-35)         | No effect (4)          |
| 500 mM NaCl    | 5           | 29 (20-35)         | Slow, partial loss of BR (3) |
| 500 mM KCl     | 8           | 31 (15-69)         | No effect (4)          |
| 10 mM SrCl₂    | 6           | 25 (21-42)         | Slow, partial loss of BR (4) |
| 10 mM BaCl₂    | 6           | 14 (10-16)         | Rapid depolymerization (6) |
| 100 mM BaCl₂   | 5           | 13 (10-18)         | No effect (6)          |
| 100 mM PIPES, pH 7.8 | 10       | 58 (30-88)        | Slow, partial loss of BR (3) |
| 100 mM PIPES, pH 5.8 | 5         | 54 (45-74)        | No effect (1)          |
| 100 mM Na-acetate, pH 4.8 | 5 | 32 (19-42) | Slow, partial loss of BR (4) |

**TABLE II**

Injection of Control Solutions

| Injected agent | No. of cells | Volume, pl (Range) | Results (no. of cells) |
|----------------|-------------|--------------------|------------------------|
| Wesson oil     | 6           | 187 (104-418)      | No effect (6)          |
| Distilled water| 11          | 53 (19-175)        | At high volumes, slow partial loss |
| 5 mM MgCl₂     | 8           | 39 (25-51)         | No effect (5)          |
| 20 mM MgCl₂    | 4           | 18 (14-29)         | Slow, partial loss of BR (3) |
| 50 mM MgCl₂    | 6           | 23 (15-35)         | No effect (4)          |
| 500 mM NaCl    | 5           | 29 (20-35)         | Slow, partial loss of BR (3) |
| 500 mM KCl     | 8           | 31 (15-69)         | No effect (4)          |
| 10 mM SrCl₂    | 6           | 25 (21-42)         | Slow, partial loss of BR (4) |
| 10 mM BaCl₂    | 6           | 14 (10-16)         | Rapid depolymerization (6) |
| 100 mM BaCl₂   | 5           | 13 (10-18)         | No effect (6)          |
| 100 mM PIPES, pH 7.8 | 10       | 58 (30-88)        | Slow, partial loss of BR (3) |
| 100 mM PIPES, pH 5.8 | 5         | 54 (45-74)        | No effect (1)          |
| 100 mM Na-acetate, pH 4.8 | 5 | 32 (19-42) | Slow, partial loss of BR (4) |

**TABLE IV**

Caffeine and Potassium Oxalate Injections

| Injected agent | No. of cells | Volume range, pl (Range) | Results (no. of cells) |
|----------------|-------------|--------------------------|------------------------|
| Caffeine       | 6           | 14-28                     | Gradient of BR loss (6) |
| Potassium oxalate, pH 6.8 | 3 | 29-42                     | No effect (3)          |
| 1 mM           | 6           | 29-51                     | No effect (6)          |
| 32 mM          | 11          | 19-48                     | Crystals formed (11)   |
| 100 mM >100*   | 220-100     | Crystals formed (all)     |

* Six cells injected while in Moore's Ca** -free seawater.

Number of cytoplasmic granules from an approximately spherical region surrounding the tip of the micropipette. The volume of the "injected region", from which the granules are displaced, is equivalent to or slightly smaller than the volume of the solution injected. After injection, granules move back into the injected region, and generally, within 1-3 s, the cytoplasm looks as it had before injection. Loss of BR induced by injected CaCl₂ (1-10 mM) is confined to part of all of the spindle in the injected region, and only in the injected region (Fig. 2).

**Slow CaCl₂ Injections**

When injected over the course of 15-60 s, large volumes of 5 mM CaCl₂ sometimes cause partial loss of spindle and astral BR, but BR is never completely abolished (Fig. 3). This result clearly contrasts with the complete and immediate obliteration of spindle BR in the injected region induced by rapid (0.25 s) injection of similar or smaller doses of CaCl₂. After slow injection, the general morphology of the spindle and asters remains unchanged, though some shortening of both spindle and asters is occasionally observed. Loss of BR, if it occurs, is always maximal at the tip of the pipette (Fig. 3).

**Controls**

Injection of >35 pl of pH buffers, monovalent salts, MgCl₂, or BaCl₂ (Table II) often causes spindle BR to diminish uniformly throughout the entire spindle during the course of a minute or more, especially when large volumes containing high salt concentrations are injected (Fig. 4). Lesser doses often have no observable effect. No rapid, local losses of spindle BR, like those observed when CaCl₂ is injected, are ever observed. Similarly, when large volumes of distilled water (up to 25% of the volume of the cell) are rapidly injected at the spindle pole, only a slight uniform reduction in spindle BR is observed (Fig. 5). Injected Wesson oil never affects spindle BR, size, or subsequent cell divisions (Fig. 6). Long term recovery from injection of high doses of salt is not uniform; recovery of spindle BR is not always complete and subsequent divisions are not always synchronous with astral rays start to regrow. Generally, regrowth is most striking when CaCl₂ is injected into the cell during prometaphase (Fig. 1), although aster and spindle fibers regrow to a limited extent even in cells injected during anaphase. After the spindles recover, cells continue to divide normally (Fig. 1). Solutions injected into these cells transiently displace a large number of cytoplasmic granules from an approximately spherical region surrounding the tip of the micropipette. The volume of the "injected region", from which the granules are displaced, is equivalent to or slightly smaller than the volume of the solution injected. After injection, granules move back into the injected region, and generally, within 1-3 s, the cytoplasm looks as it had before injection. Loss of BR induced by injected CaCl₂ (1-10 mM) is confined to part or all of the spindle in the injected region, and only in the injected region (Fig. 2).
Reversible loss of spindle BR induced by CaCl₂. (a) A prometaphase spindle of Lytechinus variegatus is shown before injection. The circle is drawn to indicate the volume of 5 mM CaCl₂ to be injected and the arrowhead marks the position of the pipette tip (not shown) during injection. (b) The injection of 16.8 pl of 5 mM CaCl₂ at the spindle pole obliterates the BR and morphology of more than half the spindle. Movement in this frame is caused by withdrawing the micropipette from the cell. Regrowth of the spindle had already begun at this early time point. (c–f) Rapid recovery to a full metaphase spindle ensues, is followed by normal anaphase (g), and development continues normally (h). Time is given in minutes from the time of injection. Bars: e, 10 μm; h, 40 μm.

CaCl₂ removes spindle BR only in a very limited region. (a and b). A Lytechinus variegatus egg at metaphase before injection. In a, the dotted white line indicates the volume of 5 mM CaCl₂ to be injected. The arrowhead marks the position of the pipette tip during injection. Inset of oil drop confirms the volume injected. In b, the same spindle without lines is shown. (c) After injection of 15 pl of 5 mM CaCl₂ at the spindle pole, only astral and some spindle BR is obliterated. The oil drop in the upper right was injected with the CaCl₂. Time is given in minutes from the time of injection. Bar, 30 μm.

SrCl₂ (10 mM) causes an immediate, local loss in spindle BR like that observed when CaCl₂ is injected. As with CaCl₂, the volume of spindle affected is equivalent to or less than the volume of 10 mM SrCl₂ injected (Fig. 7).

Ca-EGTA Injections

Ca-EGTA buffer solutions (pH 6.80, Table III) containing both 100 mM CaCl₂ and 100 mM EGTA cause losses in spindle BR that are rapid and localized, yet more extensive than the obliteration of spindle BR that is induced by CaCl₂ solutions.
Figure 3: Spindle BR is not abolished by the slow injection of CaCl₂. (a) Spindle in a late prometaphase *Lytechinus variegatus* egg. Micropipette is shown entering the cell from the upper left. The pipette was moved to the spindle pole after this photo and before injection. The circle is drawn to indicate the volume of 5 mM CaCl₂ to be injected. The arrowhead marks the position of the pipette tip during injection. (b) After the injection of 31 pl of 5 mM CaCl₂ over the course of 23 s, spindle BR remained substantially unchanged. The oil drop just above the spindle was injected along with the 5 mM CaCl₂. (c and d) Mitosis continues in a normal fashion. Time is given in minutes from the time of injection. Bar, 30 μm.

Solutions of 100 mM EGTA with 90–95 mM CaCl₂ ([Ca⁺⁺ free] = 3 – 7 μM) injected into these eggs (either rapidly or slowly), causes a more localized loss of spindle BR (Fig. 8). Within the injected region, a gradient of BR loss is observed, with maximum loss induced at the tip of the micropipette. Spindle BR does not recover as quickly or completely as after loss of BR induced by 1–5 mM CaCl₂ solutions.

When solutions containing lower concentrations of free Ca⁺⁺ are injected into these cells, no loss in spindle BR is observed (Fig. 8). When the total concentration of Ca-EGTA that is injected was reduced tenfold, to decrease by tenfold the total Ca⁺⁺-buffering capacity of the injected solution without significantly affecting the concentration of free Ca⁺⁺, no loss in spindle BR is detected.

**Caffeine Injections**

Caffeine (100 mM, pH 6.8) causes a rapid, transient loss in spindle BR. Like Ca-EGTA, and in contrast to unbuffered CaCl₂, a gradual gradient of BR loss along the length of the spindle is observed, with maximal loss at the tip of the micropipette. Spindle BR rapidly recovers and mitosis and cleavage follows (Fig. 9).

**K-oxalate Injections**

Approximately 3–5 min after eggs are injected with 32 mM or higher concentrations of K-oxalate, small, highly birefringent crystals begin to form (Table IV, Fig. 10). If only 1 or 10 mM K-oxalate is injected, no crystals form, even after several hours. When small volumes of >32 mM K-oxalate are injected, crystals form in a local region of the cytoplasm (Fig. 10 a) whose volume is approximately two to five times greater than the volume of K-oxalate injected. Larger doses (>50 pl of 100 mM K-oxalate) induce crystal formation throughout the cytoplasm (Fig. 10 b–e). Eggs continue to divide in the presence of crystals, albeit somewhat more slowly than uninjected control eggs. No variation in the distribution or number of crystals in the cytoplasm is observed as a function of stage in the cell cycle. Crystals form in unfertilized or fertilized eggs incubated with or without exogenous Ca⁺⁺.

I provide evidence that oxalate crystals are membrane-bound as follows; a clean micropipette is used to remove a sample of crystal-containing cytoplasm from an egg that had been preinjected with K-oxalate. Oil originally injected with K-oxalate is used to cap the sample and prevent its mixing with the seawater surrounding the egg. The oil and crystal containing cytoplasm
FIGURE 4 Injection of MgCl₂ does not abolish spindle BR. (a) A prometaphase Lytechinus variegatus egg before injection. Arrowhead marks the position of the pipette tip at time of injection. Inset of oil drop confirms the volume injected. (b) After injection of 29 pl of 5 mM MgCl₂, spindle BR is slightly and uniformly diminished. Small oil drop at lower pole marks site of injection. (c and d) After a transient decrease, spindle BR recovers, and the cell enters anaphase and continues to develop normally. Time is given in minutes from the time of injection. Bar, 40 μm.

are next ejected into isotonic medium (Moore's Ca²⁺-free artificial seawater [8]), supplemented with 10 mM EGTA alone or with 10 mM EGTA and 0.25% Triton X-100. Without detergent, the crystals remain intact for >30 min. In the same solution, crystals of calcium oxalate formed in vitro by mixing K-oxalate with CaCl₂ dissolved in <1 min. In solutions including 0.25% Triton, crystals formed in vivo dissolved as rapidly as those formed in vitro.

DISCUSSION

Injection calcium ions cause spindle fiber Mts to rapidly, locally, and reversibly depolymerize. The effect is Ca²⁺-specific. Of the other ions tested, only Sr²⁺ had similar effects. Other microinjected solutions (Table II) often had no detectable effect on the spindle, or, when injected at high dose, were generally toxic; BR decreased uniformly across the length and breadth of the spindle over the course of a minute or more and recovery was irregular.

Calcium depolymerized spindle fiber Mts very rapidly. If depolymerization is equated with spindle fiber Mt shortening, and a half spindle is depolymerized in <1 s (Fig. 1 is typical), observed rates of shortening are in excess of 1,000 μm/min. Such rates exceed by three orders of magnitude measured rates of chromosomal fiber shortening during mitosis (1 μm/min, [2]), or of Mt shortening induced by subunit dilution in vitro (4). The rapid rates may, however, be consistent with short half times of Ca²⁺-induced depolymerization of Mts in vitro as assayed by viscometry or light scattering (45, 73). This suggests that injected Ca²⁺ may cause structural failure of the Mt as a whole, thereby inducing loss of subunit from along the entire length of the Mt and not just from Mt ends.

Injection of Ca-EGTA buffers demonstrates that 3–7 μM free Ca²⁺ (Ca/EGTA = 0.9–0.95) is sufficient to depolymerize spindle Mts. Because the free Ca²⁺ in an injected solution of CaCl₂ is subject to rapid depletion via diffusion and endogenous Ca²⁺ sequestering or buffering agents, CaCl₂ solutions cannot accurately determine the threshold concentration of free Ca²⁺ required to abolish spindle BR. In contrast, the injected Ca-EGTA solution is an exogenous buffer that competes with cellular buffers and resists depletion of free Ca²⁺. The threshold of free Ca²⁺ required to cause muscle contraction in vivo was determined by microinjection of Ca²⁺-buffer solutions. Although 0.2–1.0 mM CaCl₂ was required to cause contraction when microinjected into living muscle fibers, as little as 0.3–1.5 μM free Ca²⁺ was sufficient when Ca²⁺-buffers were injected (49).

The concentration of free Ca²⁺ required to depolymerize Mts in vitro remains ambiguous. Salmon and Jenkins (58) and Salmon and Segall (59) found, through BR and electron mi-
croscope studies on isolated spindles, that spindle Mts depolymerized in the presence of 5–10 μM free Ca++. Keller and Rebhun (personal communication) required as little as 3 μM free Ca++ to reduce the turbidity of a 15°C preparation of reassembled sea urchin spindle Mts to one half its plateau (without Ca++) value. However, at 37°C, 300–700 μM free Ca was required. Similarly, Mts assembled from purified or partially purified Mts proteins (including tubulin and the high molecular weight proteins that copurify with it) are not consistently sensitive to Ca++. In some preparations, brain Mts are depolymerized by as little as 1–10 μM free Ca++ (19, 55, 71), whereas in others, millimolar or greater concentrations are required (45). These discrepancies may be explained by more recent experiments on the Ca++ sensitivity of brain Mts which indicate (a) that pure Mts are relatively Ca++ insensitive, (b) that calmodulin is required to confer Ca++ sensitivity to Mts in the physiological range, and that (c) the various Mts preparations may contain different amounts of calmodulin (38). Apropos to my experiments, calmodulin can be purified from sea urchin eggs (23) and is localized in the mitotic spindle of mammalian cells in culture by immunofluorescence (40, 75). It follows that the Ca++ sensitivity of spindle Mts in vivo and in isolates may not be a property of the Mts themselves, but may instead reflect the Ca++ sensitivity of a Mts-calmodulin or other accessory protein system.

Recent studies on the nucleotide requirements for in vitro polymerization of brain Mts proteins indirectly support the notion that the Ca++ sensitivity of Mts may be important physiologically. Mts polymerized in the presence of nonhydrolyzable analogs of GTP are sensitive to Ca++, even in the millimolar concentration range. This suggests that although nucleotide binding is required for Mts polymerization, nucleotide hydrolysis, aside from imparting a definite polarity to the assembling Mts (39, 70), may be essential for destabilizing Mts so as to allow their rapid depolymerization by cellular control mechanisms requiring Ca++. (72, 73).

The sensitivity of in vivo Mts to Ca++ has been evaluated in two studies using the divalent cation ionophore A23187 to increase the intracellular Ca++ concentration. In one (16), the cytoplasmic Mts of cultured mammalian cells were disassembled when cells were incubated in ionophore and 4.8 mM Ca++. In a second study (61), when specimens of the heliozoan Actinosphaerium eichhorni were incubated in as little as 10 μM Ca++ and ionophore A23187, the Mts in their axopodia were depolymerized. Although these studies are consistent with my observations on the in vivo sensitivity of Mts to Ca++, in neither study was there an attempt to quantify the cytoplasmic levels of free Ca++ that resulted from incubating cells with ionophore and various concentrations of Ca++.

In addition to demonstrating the Ca++ lability of spindle Mts in vivo, the very local obliteration of spindle BR by injected CaCl₂ suggests the existence of a strong calcium buffering or
Figure 6 Injection of Wesson oil has no effect on spindle BR. (a) A late prometaphase Lytechinus variegatus egg before injection. (b) Spindle BR and size is unaffected by the injection of 154 pl (22% of the volume of the egg) of Wesson oil. (c-f) Division in control and injected cells proceed in perfect synchrony. Time is given in minutes from the time of injection. Bar, 40 μm.

Figure 7 SrCl₂ locally abolishes spindle and astral BR. (a) Early anaphase Lytechinus variegatus egg before injection. The arrowhead marks the position of the pipette tip at time of injection. (b) After injection of 21 pl of 10 mM SrCl₂, spindle BR was almost completely obliterated in the injected region. The smaller oil drop, injected along with the SrCl₂, marks the site of injection. Time is given in minutes from the time of injection. Bar, 40 μm.

Sequestering system in these eggs. For Ca²⁺ to function in the control of Mt assembly and disassembly, a system for modulating the concentration of free cytoplasmic Ca²⁺ is required. These injection experiments indicate sequestering in three ways. (a) Spindle BR is obliterated in a highly restricted region when CaCl₂ is rapidly injected. If Ca²⁺ is freely diffusible in the cytoplasm and 1 mM CaCl₂ is sufficient to totally obliterate spindle BR in the injected region, 5 mM CaCl₂ is expected to abolish spindle BR in a larger region. Instead, loss of BR is still restricted to the injected region. The steep gradient of spindle BR caused by the injected CaCl₂ reflects a steep gradient of the Mt depolymerizing agent, Ca²⁺, for a short time after injection. Podolsky and Costantin (48) provided evidence of a Ca²⁺-sequestering system in muscle by showing that even high concentrations of Ca²⁺, microinjected into living muscle fibers, caused contraction only at the site of injection. (b) Slow microinjection of 5 mM CaCl₂ does not depolymerize spindle Mts at the tip of the micropipette. BR is only reduced, not obliterated, and spindle and aster morphology remain relatively unchanged. It seems that slow injection does not deliver sufficient Ca²⁺ to overwhelm intracellular buffers, whereas rapid injection of even 1 mM CaCl₂ supplies sufficient Ca²⁺ to depolymerize Mts at the tip of the micropipette. (c) With unbuffered Ca²⁺ solutions, nearly three orders of magnitude higher concentration of Ca²⁺ is required to depolymerize spindle Mts (see above).
FIGURE 8  Effect of injected Ca-EGTA buffer solutions on spindle BR. (a, d, and g) Three different prometaphase eggs before injection. Arrowheads mark the position of the pipette tip at the time of injection. Inset of 65-pl oil drop confirms the volume injected in g. (a–c) 188 μM free Ca$^{2+}$ (40 pl, Ca/EGTA = 1) and (d–f), 7 μM free Ca$^{2+}$ (43 pl, Ca/EGTA = 0.95) transiently abolish spindle BR in Lytechinus variegatus with maximum loss at the injected pole. Recovery is not as complete as recovery from CaCl$_2$ induced loss of spindle BR. (g–i) 0.4 μM free Ca$^{2+}$ (65 pl, Ca/EGTA = 0.5) does not abolish spindle BR in Echinarchinus parma. Time is given in minutes from the time of injection. Bar, 40 μm.
from the time of injection. Bar, 40 lam.

Rapid, extensive, and reversible loss of spindle BR induced by caffeine. (a) Spindle in Lytechinus variegatus egg before injection. The arrowhead marks the position of the pipette tip at the time of injection and the oil drop in the inset indicates the site of injection. A lone crystal is near the lower right of the cell, where the pipette entered the egg. (b–e) Oxalate crystal formation in a developing egg of Lytechinus variegatus. (b) A fertilized egg is shown in prophase of first mitosis before injection. (c) 10.8 min after injection of 65 pl of 100 mM K-oxalate (pH 6.8), small, highly birefringent crystals have begun to form. Oil drop injected with the K-oxalate is shown to the right of center, out of focus. (d) 63.6 min after injection, crystals are more numerous and are found throughout the cytoplasm. Barely visible among the highly birefringent oxalate crystals is an anaphase spindle (arrows indicate spindle poles). (e) 6 h later, the cell has divided five times, slightly retarded compared to uninjected controls. Bars, 30 μm.

These observations support a growing literature on the limited diffusion and rapid sequestration of Ca++ in cells. Restricted diffusion of injected Ca++ was observed by Rose and Lowenstein (54) in cells preloaded with aequorin, a protein that luminesces upon binding calcium. They showed that Ca-oxalate precipitates in membrane-bound vesicles in the cytoplasm of Physarum and similar Ca-oxalate deposits are localized in the endoplasmic reticulum of muscle.

K-oxalate is another probe for intracellular calcium sequestration by a vesicular fraction. Ca-oxalate precipitates have been shown to accumulate in the lumen of the sarcoplasmic reticulum, both in situ (10) and in isolated preparations (22). Ettienne (13) has demonstrated Ca-oxalate precipitates in membrane-bound vesicles in the cytoplasm of Physarum and similar Ca-oxalate deposits are localized in the endoplasmic reticulum of squid axon (26).

Numerous experiments demonstrate active Ca++ sequestration by endoplasmic reticulum fractions isolated from a variety of nonmuscle systems including platelets, adipocytes, kidney, liver, islets of Langerhans, the adrenal medulla, the pancreas, and whole brain homogenates (for review see references 6 and 7). Circumstantial physiological evidence supports Ca++ sequestration by a vesicular fraction in sea urchin egg cytoplasm (37) and Harris’ (20, 21) and Hepler’s (27) electron microscope studies have shown that numerous vesicles are found in and around the Mts of the mitotic spindle. Using the fluorescent probe chlorotetracycline, Woliński et al. (76) have recently

**Figure 10** Oxalate crystal formation in Lytechinus variegatus. (a) After the injection of 33 pl of 100 mM K-oxalate, crystal formation was restricted to a small region around the tip of the micropipette that was used to inject calcium, thereby demonstrating that Ca++ is not freely diffusible but is rapidly buffered or sequestered. In my experiments, spindle BR, not aequorin, was an indicator of free Ca++ concentration, and the very local loss of spindle BR indicates the highly restricted diffusion of injected Ca++.

Caffeine injection experiments further demonstrate vesicular Ca++ sequestration. My observations are consistent with the observation that caffeine, applied to the outside of cells, causes a rapid loss of spindle BR (50, 52). This loss of BR is not caused by caffeine’s inhibition of cyclic AMP (cAMP) phosphodiesterase (PD) and the increased concentrations of cytoplasmic cAMP that result (43, 64). Spindle BR is not lost when cytoplasmic cAMP levels in sea urchin cells are increased, even a thousandfold, by incubating the cells with cAMP or dibutyryl cAMP (44, 50, 52). Caffeine presumably diminished spindle BR through the same PD-independent mechanism by which caffeine causes skeletal muscle to contract.

Caffeine releases Ca++ from intracellular Ca++-sequestering vesicles of the sarcoplasmic reticulum by uncoupling the Ca++ pump from its ATPase (68, 69). The gradient of spindle BR that results from caffeine injection suggests that a transient gradient of caffeine, formed as caffeine diffuses away from the site of injection, causes leakage of Ca++ that locally depolymerizes spindle Mts. The immediate loss of spindle BR and the gradient of BR (maximal loss at the site of injection) that results from injected caffeine suggests that caffeine’s effect was not on the plasma membrane. Further, there is no evidence for an effect of caffeine on simple Ca++ buffering (energy-independent binding) by metabolites or other cellular constituents (proteins, etc.). Nor does caffeine induce Ca++ release from isolated mitochondria (68). Therefore, caffeine’s effects are probably caused by the uncoupling of a Ca++-sequestering system in the cytoplasm of these eggs analogous to the sarcoplasmic reticulum of muscle.

Calcium sensitivity of microtubules in vivo. This review describes the results of experiments on the effects of caffeine on spindle microtubules in sea urchin egg extracts, and reviews the implications of these results for the understanding of the role of Ca++ in the regulation of microtubule structure and function.
shown that the distribution of membrane-associated calcium coincides with the kinetochore fibers in dividing Haemanthus endosperm cells. Petzelt (46) has found Ca++-ATPase activity associated with isolated mitotic spindles and Burgess (personal communication) fractionated isolated spindles on sucrose gradients and found that the Ca++-ATPase activity was associated with a membranous fraction. Recently, Silver et al. (62) confirmed Ca++ uptake by a crude vesicular fraction associated with mitotic apparatus isolated from sea urchin embryos.

In summary, the microinjection experiments reported here show that elevated, physiological levels of free Ca++ cause the local depolymerization of spindle Mts. Furthermore, these studies suggest that there is an extensive, at least partially vesicular, Ca++-buffering system which rapidly sequesters Ca++. Thus, steep, local gradients of free Ca++, modulated by a vesicular system that appears analogous to the sarcoplasmic reticulum, may orchestrate growth and shortening of Mts in the spindle to coordinate chromosome movement during mitosis.

APPENDIX

Included in this appendix are descriptions of a new chamber in which marine eggs are immobilized for observation and microinjection, a new method for determining the volume of injected solutions, and a detailed description of a microinjection method devised, but never fully described, by Y. Hiramoto (28).

Injection Chamber for Marine Eggs

For microinjection, I immobilize viable echinoderm eggs in a chamber that allows easy access of micropipettes or other microtools to cells during observation with high resolution light microscopy. The chamber permits individual injected cells to be easily and quickly removed, then processed for electron microscopy, spindle isolation, or otherwise, as desired. To construct the chamber: (a) the long edge of a 12 × 2-mm spacer (cellophane tape or mylar, Fig. 11) is laid ~3-5 mm from and parallel to one edge of a clean glass (22 × 22 mm) coverslip. The spacer is chosen so that its thickness is ~50-70% of the diameter of the (unflattened) eggs used. (b) An 11 × 3-mm coverslip fragment is centered on top of the spacer. (c) The short edges of the fragment are fastened to the coverslip and spacer with molten Tackiwax (Central Scientific Co., Chicago, Ill.). The space underneath the fragment closest to the coverslip edge forms a rectangular injection chamber ~10-mm long, 0.3-0.8-mm deep, and as high as the thickness of the spacer (Fig. 11). The long, front side of this injection chamber remains open. Flattening the cells holds them immobile and improves the visibility of the mitotic spindle. Even if cells are not firmly held by flattening alone, they can be pushed back against the rear wall of the chamber (i.e., the spacer) and immobilized there. (d) Construction of the injection chamber is completed by drawing a line with a wax pencil along the top edge of the coverslip in front of the chamber. The chamber, fragment side down, is mounted with silicone grease (Dow Corning Corp., Midland, Mich.) across the U-shaped opening in a stainless steel or aluminum support slide (see reference 3 for basic design).

Loading Eggs into the Injection Chamber

An egg suspension is drawn into a pipette and then expelled adjacent to the lumen of the injection chamber. Capillary action draws some of the eggs and the seawater into the injection chamber. Immediately, a coverslip with a wax pencil line drawn across one edge is mounted on the underside of the metal support slide so that the wax pencil line faces the inside of the chamber directly under the wax pencil line on the upper coverslip. Rapidly, seawater is pipetted into the space between the two coverslips, up to the inside edge of the wax-pencil lines. A layer of mineral oil is added to seal the wax-pencil-line-thick opening between the two coverslips and bring the fluid level up to the edges of the coverslips. The two wax pencil lines provide a hydrophobic surface on the glass that prevents seawater from displacing the mineral oil from the front of the completed injection slide. The mineral oil acts as a cap that prevents evaporation of the seawater but allows microinstruments to pass through to the cells (Fig. 12).

Certain eggs, such as L. variegatus, must be relieved of their fertilization membranes to allow micropipette penetration. The shear between the coverslips and the eggs introduced when capillary action draws the eggs into the chamber can be used to remove the fertilization membrane, provided the fertilization envelope has risen fully but has not yet hardened. Almost all L. variegatus eggs loaded into the injection chamber between 4 and 8 min after fertilization are demembranated. Thus, no separate demembranation technique was required.

Unfertilized eggs, flattened and immobilized in the chamber, can be fertilized by adding sperm to the seawater in the injection slide. Whether fertilized before or after being loaded into the chamber, eggs divide normally and synchronously. After hatching, embryos swim out of the injection chamber.
FIGURE 12  Preparation and loading of an injection chamber for immobilizing and viewing cells. (a) A spacer is placed parallel to the edge of a coverslip. (b) A coverslip fragment is centered on spacer, and (c) cemented into place. (d) Next, the chamber is sealed by the surface tension of silicone grease to a stainless steel support slide. (e) Cells are drawn into the injection chamber by capillary action as they are pipetted along the front side of the chamber. (f) The finished chamber; a bottom coverslip was sealed into place, the preparation was filled with seawater, and the open end was capped with mineral oil. The arrows in c, d, and e indicate the wax pencil line. Details are given in the Appendix, under “Injection Chamber for Marine Eggs.”

and into the large lumen of the injection slide where they mature normally.

Microinjection System

Hiramoto’s injection system (28) consists of a pulled glass micropipette, a small drop of mercury, a micropipette holder, pressure-tight connecting tubes, and a screw advance glass syringe. Its unique feature is a simple, yet elegant pressure-volume transducer, that allows the small, precise volume changes required for gentle but rapid microinjection. The screw driven, fluid filled syringe (1–5 mls) is used to increase, maintain, or decrease the pressure on the small drop of mercury placed in the neck of the micropipette. The mercury, its high surface tension tending to minimize its surface area, tries to form a sphere and backs into the shaft of the micropipette, away from the tip. Applied pressure forces the mercury towards the tip. Changes in pressure, effected by manipulating the screw driven syringe, alter the equilibrium position of the mercury, causing it to advance or retreat from the tip. Thus, relatively large changes in volume in the screw driven syringe are indirectly but precisely coupled (through the mercury-pressure volume transducer) to the minute volume changes at the micropipette tip.

The rate at which pressure is applied to the mercury can be altered by varying the total volume of gas included in the microinjection system. If a large volume of air is included, advance of the piston in the screw driven syringe develops pressure only slowly, and the volume change at the pipette tip is consequently small. If the volume of air in the syringe is decreased, pressure is rapidly developed and volume changes in the pipette tip are great. Precise control of volume changes ranging from <0.01 pl to >1 nl can be accomplished by choosing an appropriate volume of included air. The system, therefore, has a wide range of microinjection applications.

Assembly of the Microinjection Apparatus

A sufficiently pressure-tight system is constructed of inexpensive, easily modified components: glass micropipettes are drawn from 0.8-mm outside diameter capillary stocks (Drummond Scientific Co., Broomall, Penn.) on a Leitz DuBois, (E. Leitz, Inc., Rockleigh, N. J.) micropipette puller (12). A small drop of nitric acid cleaned mercury (drop diameter slightly greater than the inside diameter of the shaft of the micropipette) is deposited in the shaft of the micropipette. The pipette is friction fit into a Frank and Chambers type micropipette holder (E. Leitz, Inc. [9]), the rear end of which is threaded and fit to a tapped male Luer lock adapter (Clay Adams no. A7550; Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). Connecting tubes consist of polyethylene tubing (PE 20, Clay Adams) fitted on each end with a female Luer lock fitting (Clay Adams no. A7540). A 1-cm³ glass tuberculin syringe (B-D and Yale, Div. of Becton, Dickinson & Co., Rutherford, N. J.) with a male Luer lock fitting is mounted on a screw driven syringe holder (E. Leitz, Inc.). To insure against pressure leaks, fittings are greased with high vacuum silicone grease (Dow Coming Inc.) before joining them. Before fitting the micropipette, the system is filled with an appropriate hydraulic fluid. Cavitation under the influence of “negative” pressure is minimized by choosing a fluid with low vapour pressure and a low surface tension (to most efficiently wet internal surfaces and minimize nucleation points at which the cavitation-phase change most readily occurs). It is convenient if the hydraulic fluid is also nontoxic. Boiled deionized water, or better, degassed fluorocarbon oil (FC-47, Grand Island Biological Co., Grand Island, N. Y.) proves effective. Operationally, sufficient control for injection of volumes ranging from 0.1 to 100 pl into marine gametes is afforded by completely filling the system with FC-47 oil and allowing the only gas in the system to be that air introduced in the shaft of the micropipette.
The micropipette holder is fitted to a micromanipulator for positioning during pipette loading and microinjection. All subsequent operations are performed during observation with the ×20 or ×40 objective on the microscope used for microinjection.

**Filling the Micropipettes**

Micropipettes are filled through their tips, just before injection, from a glass capillary reservoir containing ~4 μl of injection solution capped at the proximal end with 0.5–1 μl of a nontoxic vegetable oil to prevent evaporation. I used fresh Wesson oil throughout my studies; it proved nontoxic to living cells (Fig. 6) and did not denature isolated proteins, such as aequorin or γ-globulin. The distal end of the reservoir is sealed with petroleum jelly or silicone grease. The filled reservoir is mounted directly on the injection slide. Because the lumen of the micropipette as pulled is too fine for liquids, or even air, to flow through, its tip usually has to be removed. The fragile tip is pushed gently against the end of the capillary reservoir to break it and obtain a 0.5–1 μm (inside diameter) pipette. Next, pressure is applied to drive the mercury to the very tip of the micropipette. The reservoir is subsequently maneuvered to position the pipette tip first in the oil and then in the aqueous solution. Equal volumes of each are loaded into the pipette by sequentially allowing each solution to flow into the pipette the same linear distance (from the tip). This oil prevents direct contact between the aqueous solution and the mercury and allows determination of injected volume (after injection, the oil droplet is expelled into the seawater, its diameter measured, and its volume calculated). The aqueous injection solution is subsequently capped with oil by repositioning the pipette tip in the oil phase of the reservoir and loading a second volume of oil. This oil cap prevents evaporation from the tip and contamination of the contents of the micropipette by the culture medium bathing the cells. This process could be repeated until five or more doses of aqueous solution, separated by oil spacers, were loaded into a single pipette.

Micropipettes as pulled varied in bore and taper (though often only very slightly). If it is desirable to load precisely predetermined volumes into the micropipette, each pipette can be precalibrated with oil that is subsequently ejected into distilled water.

**Microinjection of Echinoderm Eggs**

The fully loaded pipette and the target cells in their injection slide are next positioned and centered in the microscope field. Final focusing requires the shallow depth of field of the ×40 objective, which assures vertical alignment of the cell and the micropipette. When large volumes of aqueous solution are to be injected (>1% of the volume of the egg), an equal volume of Wesson oil is not injected. Although nontoxic, large volumes of oil tend to obscure structures and processes of interest. Consequently, most of the oil cap is expelled into the seawater surrounding the cells. Once all but a tiny drop of oil (optimally <1 pl) is expelled from the tip, the micropipette is ready for microinjection. The pipette is positioned normal to the edge of the cell, then advanced further to depress the cell surface; the pipette is still not inside the cell. After the pipette is pushed approximately one-fourth to one-third the way across the cell, the deformed cell periphery snaps out around the pipette to regain its circular outline. At this time, the pipette is usually, but not always, inside the cell. To insure penetration, pressure was released until a small amount of cytoplasm flowed into the pipette.

Positive pressure is then applied to microinject the small oil cap and the aqueous solution into the cell. Often, the injected oil droplet sticks to the end of the pipette, so the site of injection is not always marked by its position. After injection, rapid withdrawal of the pipette seems to cause the least damage to the cell.

If a pipette is loaded with several doses of solution, most of the spacer oil is expelled into the seawater and the microinjection process is repeated on another cell.

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