Calcium Restriction Prolongs Metaphase in Dividing *Tradescantia* Stamen Hair Cells

PETER K. HEPLER
Department of Botany, University of Massachusetts, Amherst, Massachusetts 01003

ABSTRACT Agents that lower extracellular calcium concentration (EGTA) or modulate calcium transport (lanthanum or D600) have been applied to dividing stamen hair cells of *Tradescantia* and analyzed for their ability to change the following: (*a*) the time required to progress from nuclear envelope breakdown to the onset of anaphase (metaphase transit time), (*b*) the time required to progress from anaphase to the initiation of the cell plate, and (*c*) the rate of chromosome motion in anaphase. Control cells complete metaphase in 32 min, initiate a cell plate in 19 min, and display a chromosome motion rate of 1.45 μm/min. If cells are treated with a calcium-EGTA buffer (pCa 8) for 4 h, the metaphase transit time is increased to 53 min without any change in the time of cell plate formation or the rate of chromosome motion. Lanthanum and D600, under conditions in which their access to the plasmalemma has been facilitated by pretreating the cells with cutinase, also markedly extend metaphase and in several instances permanently arrest cells. Lanthanum, however, produce little or no change in cell plate initiation or the rate of chromosome motion. Microscopic observations of the mitotic apparatus in calcium-stressed cells reveal normal chromatin condensation and metaphase progression. Chromosomes partly untwine but remain attached at their kinetochores. It is suggested that a flux of calcium, derived from the extracellular compartment, may cause the final splitting of sister chromsomes and trigger the onset of anaphase. However, once anaphase has begun, chromosome motion and cell plate initiation proceed normally even under conditions of extracellular calcium restriction.

It is generally thought that fluxes in calcium ion concentration ($[Ca^{2+}]$) contribute to the regulation of mitosis. Beyond the well-established role of calcium in mitogenesis (18, 32), several converging lines of inquiry suggest that calcium participates during chromosome motion. For example, an elevated $[Ca^{2+}]$ causes disassembly of spindle microtubules in vitro (25) and in vivo (16), and enhances movement of chromosomes to the poles (2, 8). The calcium regulatory components including calmodulin (31, 37), Ca^{2+} ATPase (20, 21), and an extensive membrane system (10) have been shown to be present in the mitotic apparatus (MA).[1] Physiological studies further demonstrate that the mitotic membranes are capable of sequestering calcium (27). Together these studies provide good circumstantial evidence for a role of calcium in mitosis; however, the specific time at which the ion works or whether fluxes in $[Ca^{2+}]$ normally occur have been difficult to determine.

Recent microscopical and physiological studies have begun to elucidate a plausible temporal scheme for calcium action by providing evidence for ion changes and sensitivity at the metaphase/anaphase transitions. Wolniak et al. (36), using the calcium membrane probe chlorotetracycline, noted a reduction in fluorescence a few minutes before the onset of anaphase. These observations indicate that the membrane-associated calcium had declined and suggested that the free $[Ca^{2+}]$ had increased. In addition, two charge-sensitive probes, aniline naphthaline sulfonate and dipentyl dioxacarbacyanine, showed large increases in fluorescence beginning precisely at the onset of anaphase. The temporal coordination of these fluorescence changes with the events of mitosis, and the fact that they were spatially confined to the MA, provide evidence for ion redistribution, including possibly calcium, in the MA at the time of mitosis. The direct determination of $[Ca^{2+}]$ is being attempted by different laboratories using the fluorescent indicators quin-2 (6, 14, 35) and fura-2 (22), and preliminary results indicate that the cytoplasmic-free concentration of the ion changes during mitosis.

[1] *Abbreviation used in this paper:* MA, mitotic apparatus.

Further evidence that calcium is involved in the metaphase/anaphase transition comes from different physiological studies. Izant (12), using PtK cells, has reported that a lowered intracellular [Ca^{2+}] brought about by microinjection of an EGTA-Ca^{2+} buffer (pCa 7.3) retards the onset of anaphase. The calmodulin antagonists chloropromazine (1), W-7, calmidazolium, and trifluoperizine (15) also retard the onset of anaphase or arrest cells in metaphase. These studies suggest that lowering intracellular [Ca^{2+}] or blocking calmodulin activity prolong metaphase. Contrasting views have been presented, however, by Chai and Sandberg (4) who report that restriction of extracellular calcium with EGTA or lanthanum promotes the progression of Chinese hamster DON cells from metaphase to telophase, and by Chafouleas et al. (3) who note that the calmodulin antagonist W-13 has no effect on mitosis in Chinese hamster ovary cells.

Attempts to modulate mitosis with elevated levels of calcium have also yielded conflicting results. An excess of calcium in the medium, for example, has been reported to inhibit Chinese hamster DON cell progression from metaphase to telophase (4). Similarly, the application of the calcium ionophore, A23187, prolongs metaphase in HeLa cells (28, 29). However, A23187 plus exogenous calcium, under conditions that promote hydrogen ion efflux and hence calcium influx, have no effect on mitosis in spermatogenous cells of *Marsilea* (33). A more direct approach to the effect of high intracellular [Ca^{2+}] on mitosis has come from the recent study of Izant (12) who has microinjected citrate-glutamate-buffered solutions of calcium into PtK cells and has reported that 1.0 μM free calcium hastens the onset of anaphase. Unfortunately, it is not known how cells injected with a resting level of calcium (0.1 μM) behave, and thus there is a question of whether the cells with 1.0 μM calcium are hastened through metaphase or are simply proceeding at a normal rate.

The role of calcium during mitosis is thus far from settled. Depending on the particular study, increasing or decreasing the [Ca^{2+}] can either retard or advance anaphase onset. The results presented herein indicate that decreasing the extracellular concentration with EGTA or inhibiting the transport of calcium ions with lanthanum of D600 in stamen hair cells of *Tradescantia* prolongs metaphase. However, if the cell enters anaphase, its subsequent chromosome motion and initiation of cell plate occur normally. These studies direct attention to the plasma membrane as contributing to the regulation of calcium in the MA.

These results were presented at the Cell Biology Meetings of 1983 (9).

MATERIALS AND METHODS

Dividing stamen hair cells of the flowering plant *Tradescantia virginiana* were used throughout the study. The plants were obtained from the Botanical Garden at the University of Sydney, Australia, and were cloned and propagated vegetatively under controlled conditions (16-h day/8-h night) in growth chambers.

Dividing hair cells were usually found in the fifth or sixth bud back from the one that was flowering. The base of the bud was excised with a razor and the ovary and attached stamens were gently squeezed out into a few drops of control culture fluid (HEPES, 20 mM [Sigma Chemical Co., St. Louis, MO]; KCl, 20 mM, pH 7.0). The anthers were removed and the stamen filaments with attached hairs were rinsed briefly (1–2 min) in 0.05% Triton X-100 (Sigma Chemical Co.) to facilitate wetting of the waxy cuticle, and then placed in their respective culture solution as follows: (*a*) control (HEPES, 20 mM; KCl, 20 mM, pH 7.0); (*b*) pCa 5 (control plus EGTA, 10 mM [Sigma Chemical Co.]; $CaCl_2$, 9.64 mM); (*c*) pCa 5/pMg3 (pCa5 plus $MgCl_2$, 1.01 mM); (*d*) pCa 8/pMg3 (control, plus EGTA, 10 mM; $CaCl_2$, 0.25 mM; $MgCl_2$, 1.3 mM); (*e*) lanthanum (HEPES, 20 mM; KCl, 20 mM, pH 6.5, plus $LaCl_3$, 0.1 mM); (*f*) D600 (Knoll Pharmaceutical Co., Whippany, NJ) (control plus D600, 0.5–20 μM); or (*g*) cutinase: (HEPES, 20 mM; KCl, 20 mM, pH 8.0, plus cutinase, 0.1 mg/ml).

Stamen hairs were cultured in small plastic petri dishes in the respective solutions for ~1 h before examination in the light microscope. In the initial studies with EGTA, it was necessary to culture the cells for 4 h before microscopic examination in order to deplete the wall-bound calcium. However, after pretreatment with cutinase, a fungal enzyme obtained from Drs. P. Kolattukudy and C. Soliday at Washington State University (Pullman, WA), the extracellular calcium could be removed more easily and a long soak was not required.

After incubation, the stamen hairs were mounted in simple slide-cover glass chambers and examined in the light microscope using Nomarski differential interferences-contrast optics. Cells in late prophase were identified and followed from nuclear envelope breakdown through cell plate formation. Many observations were made directly, although several sequences were recorded on film, including both 35-mm and 16-mm time-lapse cinematography. Subsequently, mitotic cells were recorded by time-lapse video microscopy. In this instance, an SIT camera was used to permit exposure of cells to relatively low levels of light and thus to reduce the possibility of photodamage.

Measurements were made on (*a*) the time required to progress from nuclear envelope breakdown to the onset of anaphase (metaphase transit time), (*b*) the time required to progress from anaphase to the initiation of the cell plate, and (*c*) the average rate of chromosome motion during the first 10 min of anaphase. The data are presented as the mean ± two standard errors of the mean.

RESULTS

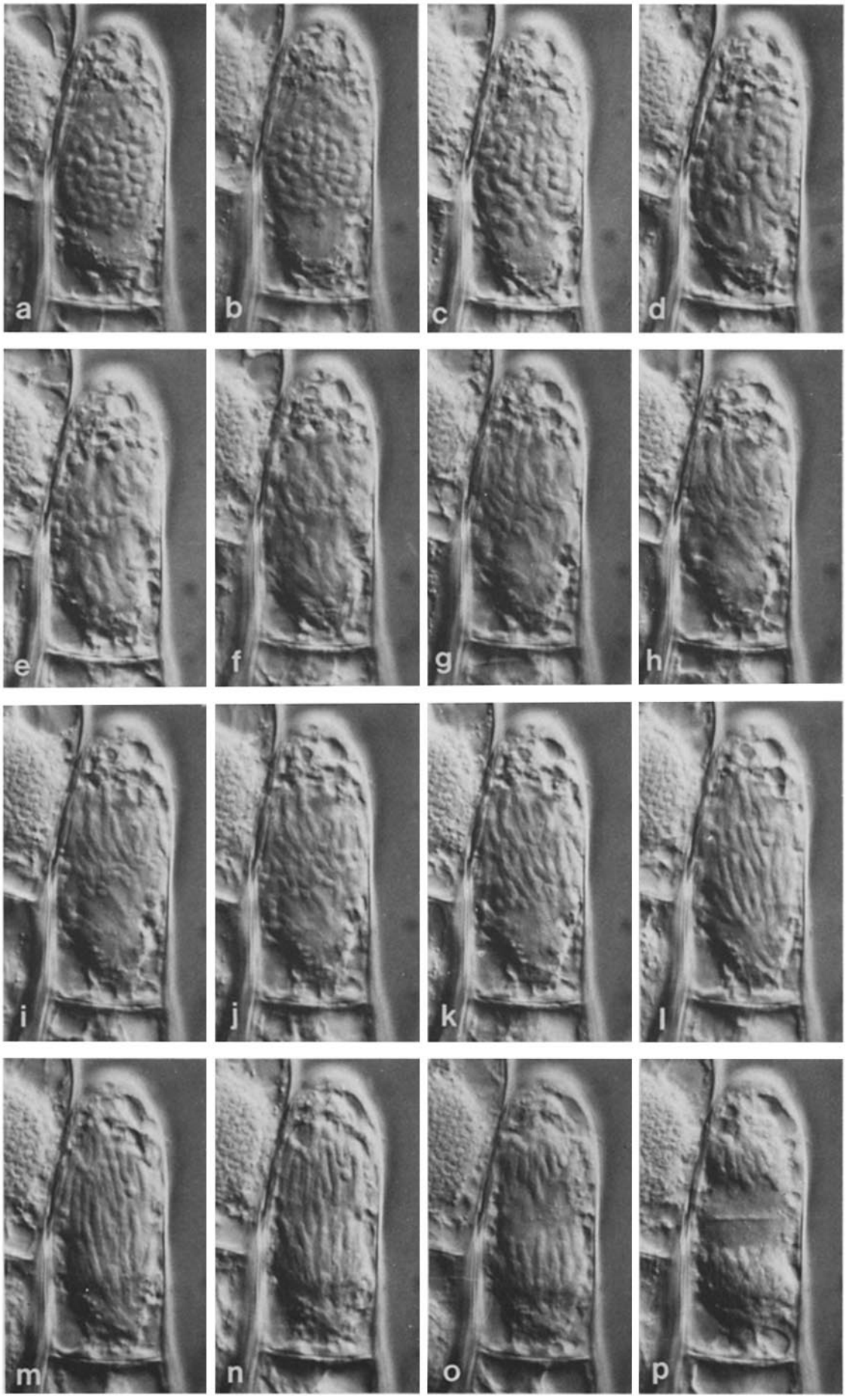
A typical example of mitosis in a hair cell of *Tradescantia* is shown in Fig. 1. Fig. 1 *a* shows the cell at the moment of nuclear envelope breakdown. The remaining figures (*b–p*) are a time-lapse sequence of this cell throughout division (see figure legend for time intervals). This particular cell required 29 min (Fig. 1 *i*) to progress from nuclear envelope breakdown to the onset of anaphase, an additional 23 min to initiate a cell plate (Fig. 1 *p*), and moved its chromosomes at a rate of 1.42 μm/min.

Metaphase Transit Time

Cells cultured in the control buffer require an average of 32.5 min to progress from nuclear envelope breakdown to the onset of anaphase (Table I). Even after cells are cultured for up to 7 h, this time remains constant. When the cells are cultured in control buffer plus 10 mM EGTA (free [Ca^{2+}] at approximately 1×10^{-8} M) (pCa 8/pMg3), there is at first no effect. After 4 h of culture, however, the metaphase transit time is increased to 52.6 min, and a few cells appear to be permanently arrested in metaphase (Table I). When the cells are cultured in the absence of magnesium, there appear to be no changes from controls in metaphase transit time. Even so, magnesium (1 mM) has been included in the preparations with low calcium to guard against deleterious affects that might occur during long-term culture of cells in the absence of this ion. Cells cultured in pCa 7 or higher concentrations of calcium show no prolongation of metaphase. Lanthanum (La^{3+}) and D600 in the initial experiments seemed to have little or no effect except that a few cells in D600 became permanently arrested in metaphase (Table I).

In the course of these studies I came to suspect that the cuticle, which forms a continuous waxy layer on the outer

FIGURE 1 A time-lapse sequence of mitosis in a stamen hair cell of *Tradescantia* from nuclear envelope breakdown (a) to cell plate initiation (p). Times, in minutes, after nuclear envelope breakdown, are given as follows: (a) 0, (b) 2, (c) 4, (d) 8, (e) 12, (f) 16, (g) 24, (h) 28, (i) 29, (j) 30, (k) 32, (l) 36, (m) 40, (n) 44, (o) 48, and (p) 52 min. × 680.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surface of the wall, might be impeding the passage of molecules or even ions to the plasmalemma. In an attempt to alleviate this problem, the hairs were pretreated with cutinase. This enzyme appears to etch the cuticle and facilitate transfer of solutions through the cell wall to the plasma membrane. Under these conditions, the EGTA-buffered calcium (pCa 8/pMg3) caused its prolongation of metaphase within the first hour. I also found that pCa 7 had little or no effect and that pCa 7.5 was intermediate between pCa 7 and 8. Of additional interest is the finding that lanthanum and D600 markedly prolongs metaphase transit time (Table I). Some cells in La^{3+} (0.100 mM) and most of the dividing cells in D600 (1–20 μM) become arrested in metaphase. When the D600-arrested cells are given calcium, they progress through mitosis. If D600 is given together with 2 mM $CaCl_2$, the cells progress through metaphase in normal time (Table I).

TABLE I
Metaphase Transit Time

Treatment	Time (min)	Number of cells
Noncutinase		
Control	32.5 ± 3.9	20
pCa 5	35.8 ± 5.1	6
pCa 5/pMg3	35.2 ± 5.4	6
pCa 8/pMg3	52.6 ± 6.6	17*
La^{3+}	32.0 ± 6.0	6
D600	38.5 ± 6.6	12*
Cutinase		
Control	32.4 ± 2.9	9
pCa 7/pMg3	34.5 ± 1.0	2
pCa 8/pMg3	54.5 ± 19.0	4*
La^{3+}	50.3 ± 5.8	5*
D600	>120.0	14‡
D600/pCa 3	36.0 ± 8.8	7*

The data are expressed as the mean ± two standard deviations of the mean.
* Not included in the mean are two cells in each instance that were arrested in metaphase longer than 120 min.
‡ Not included in the mean are two cells that entered anaphase in less than 120 min (97 min and 46 min).

The morphology of cells prolonged in metaphase is quite similar between the different treatments; an example of a cell cultured in pCa 8/pMg3 is shown in Fig. 2. After nuclear envelope breakdown, the chromosomes appear to condense and contract normally. As they become unwound, however, the final splitting of the chromatids at their kinetochore attachment is retarded or it fails. Many cells with prolonged metaphase have been examined in which it seemed certain that the chromatids were essentially ready to split, yet the final act would not occur. Fig. 3 shows this point; in Fig. 2*a* the cell is shown 45 min after nuclear envelope breakdown. The chromosomes, indistinguishable from normally condensed chromosomes, are on the verge of separating, this sequence shows, however, that the split does not occur until 52 min (Fig. 2*c*).

A feature of some calcium-stressed cells, not shown in the above sequences, is that the chromosomes and surrounding spindle often lose their optical refractive index difference and become glazed in appearance. It may indicate that the chromatin is beginning to disperse due to a lower internal $[Ca^{2+}]$. It is not simply the result of cell necrosis since glazed-appearing cells will later enter and complete anaphase normally, and form a cell plate.

It is additionally pertinent to note at this point that the issue of cell death due to the various treatments was taken into account throughout the study. Thus, if a particular cell under investigation acquired either a highly refractile-appearing cytoplasm or large spherical vacuoles, and also showed Brownian motion of the small cytoplasmic particles, it was discarded. In addition, a dividing cell was discarded from analysis if its nondividing neighboring cell(s) failed to show cytoplasmic streaming. An attempt was thus maintained throughout this study to obtain results only from healthy cells residing in healthy stamen hairs.

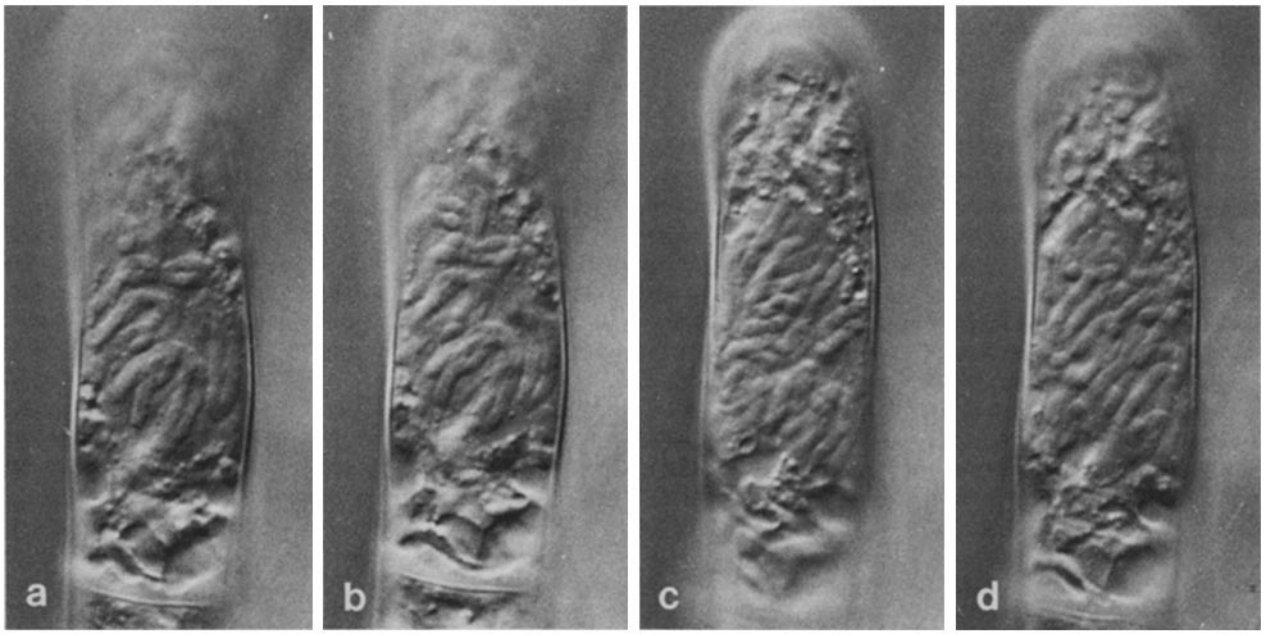

FIGURE 2 A time-lapse sequence showing the metaphase/anaphase transition in a cell cultured in pCa8/pMg3. Times, in minutes, after nuclear envelope breakdown, are given as follows: (a) 45, (*b*) 50, (c) 52, and (*d*) 56 min. × 880.

TABLE II

Time of Cell Plate Initiation

Treatment	Time (min)	Number of cells
Control	18.9 ± 1.1	18
pCa 8/pMg3	19.6 ± 1.7	5
La^{3+}	22.0 ± 4.8	4

The data are expressed as the mean ± two standard deviations of the mean.

TABLE III

Rate of Chromosome Motin

Treatment	Rate (μm/min)	Number of cells
Control	1.45 ± 0.043	18
pCa 8/pMg3	1.48 ± 0.095	6
Cutinase control	1.34 ± 0.084	8
La^{3+}	1.18 ± 0.096	4

The data are expressed as the mean ± two standard deviations of the mean.

Time of Cell Plate Initiation

The time required to progress from the onset of anaphase to the appearance of a perceptible phase-dense cell plate is 19 min in cells cultured under control conditions (Table II). Only slight increases in this time are brought about by culture in pCa 8/pMg3 and lanthanum. Because so few cells in D600 entered anaphase, it has not been possible to assess the effect of this agent either on time of cell plate formation or chromosome motion (see below).

Rate of Chromosome Motion

Control cells exhibited a chromosome motion of 1.45 μm/min. pCa 8/pMg3 has no effect on this process (Table III). Cutinase pretreated controls exhibited a slightly slower motion than noncutinase controls. Although lanthanum retards the rate further below cutinase controls, the magnitude is small, and it is questionable whether a biological significance can be concluded from the data.

DISCUSSION

Restricting calcium entry or modifying calcium transport during mitosis in stamen hair cells of *Tradescantia* markedly prolongs metaphase transit time. However, two of these agents (EGTA and La^{3+}), which have a pronounced effect on metaphase, show little or no modulation of the subsequent stages of mitosis including the time of cell plate initiation and anaphase movement of the chromosomes.

The results presented herein thus agree with those of Izant (12) which show that PtK cells microinjected with an EGTA buffer containing less than 0.1 μM free calcium are retarded from entering anaphase. However, there is considerable disagreement with the published results of Chai and Sandberg (4), which show that the chelators EDTA and EGTA and the calcium antagonist lanthanum all accelerate the progression of cells through metaphase. The explanation that underlies the differences in results is unknown. Before conclusions can be made, it will be important to determine the intracellular $[Ca^{2+}]$ after these various treatments. One cannot conclude on the basis of modulation of extracellular $[Ca^{2+}]$ that the intracellular level has changed in a similar way.

The studies reported here are consistent with the notion that calcium entry into the cell from the extracellular wall space is necessary for cells to progress from metaphase to anaphase. All the agents used appear to restrict or block calcium extracellularly. EGTA does not appear to cross the plasma membrane; due to its charge, size, and hydrophilicity, the chelator probably remains in the wall space and extracellular fluid where it would be expected to create an abnormally low $[Ca^{2+}]$. Thus, an influx of calcium would be reduced or prevented. Lanthanum achieves its effect by competing for calcium binding sites (17, 23). It also does not cross the plasma membrane, as has been shown directly with electron microscopy (30). By tightly binding to the calcium transport complexes, and displacing the ion without itself being transported, lanthanum creates a condition in which calcium would fail to flow into the cell.

D600 is reported to inhibit the slow, voltage-dependent calcium channel (7, 13). Studies on cardiac and smooth muscle cells show that it and related agents are specific for calcium transport at the sarcolemma and have little effect on the sarcoplasmic reticulum (7, 13). Hescheler et al. (11) suggest that D600 permeates the cardiac sarcolemma and inhibits calcium transport by acting on the inside of the membrane. Thus by different mechanisms EGTA, lanthanum, and D600 all appear to restrict or block calcium influx from the extracellular space. The results obtained with these agents support the idea that the plasma membrane in *Tradescantia* regulates the calcium transients that trigger the final separation of the chromosomes and initiate anaphase.

It has come as a surprise during these studies that the various calcium-perturbing agents have little or no effect on chromosome motion and time of cell plate formation. As a regulator of microtubule depolymerization, one expects calcium to modulate the rate at which chromosomes move to the poles; specifically, low $[Ca^{2+}]$ might reduce the rate of anaphase motion. Cell plate formation is also thought to be a calcium-requiring process; calcium deposits occur in abundance in the plate (26, 34), and low $[Ca^{2+}]$ is reported to inhibit cell plate formation in roots (19). However, the studies with pCa 8/pMg3 culture medium demonstrate that cells, which are undeniably stressed by virtue of their prolonged metaphase, nevertheless move their chromosomes at control rates and initiate and complete a cell plate at the normal time after the onset of anaphase.

In view of the results showing that supposed calcium-sensitive processes are unaffected by modulation of the $[Ca^{2+}]$, it might be tempting to suggest that events such as chromosome motion and cell plate formation are not regulated by this ion. However, such a conclusion would be premature. Although the experimental conditions may have severly reduced the $[Ca^{2+}]$ in the vicinity of the plasma membrane or blocked its transport into the cell, we know nothing of the ion concentration in the MA itself. Local domains within the cell or the MA brought about by an extensive, ramifying endomembrane system could create selected areas in which the $[Ca^{2+}]$ is spatially regulated. Until we measure the free $[Ca^{2+}]$ within the cell or within subregions of the cell, we are unable to conclude, simply as a function of perturbation of the ion outside of the cell, what the internal concentrations are.

Although we are unable to resolve whether chromosome motion and cell plate initiation are calcium-requiring processes, we can assert that the onset of anaphase is a calcium-dependent event. The results suggest that an influx of calcium

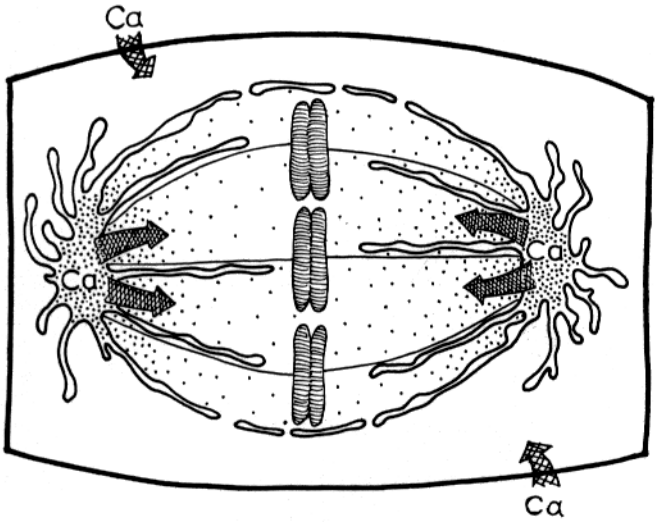

FIGURE 3 A diagrammatic representation of a cell in late metaphase. The inner part of the figure, from Hepler et al. (12), shows the spindle apparatus and its associated endomembranes. The original figure has been modified here to include the plasma membrane. It is suggested that a calcium influx across the plasma membrane regulates the separation of chromosomes and the onset of anaphase. Either the calcium influx directly stimulates chromosome splitting or it induces a further release of calcium from the endomembrane system that triggers the onset of anaphase.

across the plasma membrane during late metaphase triggers the separation of the chromosomes and marks the beginning of anaphase. The process is regulated in such a way that all chromosomes split at exactly the same time. In *Tradescantia* cells, prolonged in metaphase by calcium stress, it seems pertinent to note that if the chromosomes separate they do so at the same moment. No example has been observed in which chromosomes separated at different times, or in which some separated but others did not. Chromosome separation in *Tradescantia* thus appears to be an all-or-none process that is triggered by increases in $[Ca^{2+}]$ that exceed a minimum threshold level.

The results showing that conditions which markedly prolong metaphase have little or no effect on anaphase and cell plate initiation suggest that later processes are not regulated at the plasma membrane. It seems reasonable to conclude that if calcium is necessary for the subsequent events, the pulse which finally triggers chromosome splitting is sufficient to support normal anaphase motion and cell plate initiation. The calcium solely derived from the plasma membrane may be enough for this task or, perhaps more likely, the calcium from the plasma membrane stimulates a larger release of calcium from internal stores, and that this latter event constitutes the basis for control of spindle function and cytokinesis. Calcium-induced calcium release mechanisms have been well-established for the regulation of heart muscle contraction (5) and may participate in other cell types as well (24). These thoughts are summarized in Fig. 3, which schematically depicts a *Tradescantia* cell in late metaphase. A small calcium influx across the plasma membrane causes a release of calcium from the spindle-associated membrane system. This latter step induces the final separation of the chromosomes and may regulate anaphase and cytokinesis.

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