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The Timing of Protein Kinase Activation Events in the Cascade That Regulates Mitotic Progression in *Tradescantia* Stamen Hair Cells

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Stamen hair cells of the spiderwort plant *Tradescantia virginiana* exhibit unusually predictable rates of progression through mitosis, particularly from the time of nuclear envelope breakdown (NEBD) through the initiation of cytokinesis. The predictable rate of progression through prometaphase and metaphase has made these cells a useful model system for the determination of the timing of regulatory events that trigger entry into anaphase. A number of studies suggest that the elevation of one or more protein kinase activities is a necessary prerequisite for entry into anaphase. The current experiments employ two strategies to test when these elevations in protein kinase activity actually occur during metaphase. In perfusions, we added the protein kinase inhibitors K-252a, staurosporine, or calphostin C to living stamen hair cells for 10-min intervals at known times during prometaphase or metaphase and monitored the subsequent rate of progression into anaphase. Metaphase transit times were altered as a function of the time of addition of K-252a or staurosporine to the cells; metaphase transit times were extended significantly by treatments initiated in prometaphase through early metaphase and again late in metaphase. Transit times were normal after treatments initiated in mid-metaphase, ~15 to 21 min after NEBD. Calphostin C had no significant effect on the metaphase transit times. In parallel, cells were microinjected with known quantities of a general-purpose protein kinase substrate peptide, VRKRTLRRL, at predefined time points during prometaphase and metaphase. At a cytosolic concentration of 100 nM to 1 μM, the peptide doubled or tripled the metaphase transit times when injected into the cytosol of mitotic cells within the first 4 min after NEBD, at any point from 7.5 to 9 min after NEBD, at any point from 14 to 16 min after NEBD, at 21 min after NEBD, or at 24 min after NEBD. At the concentration used and during these brief intervals, the peptide appeared to act as a competitive inhibitor to reveal inflection points when protein kinase activation was occurring or when endogenous substrate levels approached levels of the peptide. The timing of these inflection points coincides with the changes in protein kinase activities during prometaphase and metaphase, as indicated by our perfusions of cells with the broad spectrum kinase inhibitors. Collectively, our results suggest that the cascade that culminates in anaphase is complex and involves several successive protein kinase activation steps punctuated by the activation of one or more protein phosphatases in mid-metaphase.

INTRODUCTION

During the past several years, there has been a significant improvement in our understanding of the regulatory pathway that mediates progression through the cell cycle. Perhaps in all eukaryotes a major player in this cascade is the protein kinase whose catalytic subunit is known as $p34^{cdc2}$, which, with its regulatory cyclin subunits, controls entry into mitosis from the G_2 portion of interphase (Draetta and Beach, 1989; Lohka, 1989; Maller et al., 1989; Moreno et al., 1989; Norbury and Nurse, 1989, 1992; Wolniak, 1991; Jacobs, 1992; John et al., 1993). The signaling mechanisms that regulate progression from metaphase to anaphase may also involve $p34^{cdc2}$, but as metaphase progresses, it probably acts in a subsidiary role to other protein kinases, to protein phosphatases (Booher and Beach, 1989; Vandre and Borisy, 1989; Axton et al., 1990; Pondaven, 1991; Wolniak and Larsen, 1992; Zhang et al., 1992; Strausfeld et al., 1994), and to calcium, whose transient increases in activity during prometaphase and metaphase (Wolniak, 1988; Hepler, 1989, 1994) contrast sharply with the inactivation of $p34^{cdc2}$ by cyclin degradation at or around the time of anaphase onset (Norbury and Nurse, 1989, 1992; Holloway et al., 1993; Suprynowicz, 1993; Suprynowicz et al., 1994).

We have focused on the regulation of mitotic progression in intact stamen hair cells of the spiderwort plant *Tradescantia virginiana* because these cells exhibit unusual predictability in their metaphase transit times, requiring 33 min ± 2 min (SD) to progress from nuclear envelope breakdown (NEBD) to anaphase onset (Hepler, 1985; Wolniak and Bart, 1985a,

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1985b). During the first 6 to 10 min of this 33-min interval, when the cells are in prometaphase, the replicated chromosomes undergo congressional movement toward the spindle equator. Thereafter, the cells have entered metaphase, and the chromosomes reside at the metaphase plate.

Our earlier work has focused on the regulatory events responsible for entry into anaphase. Our results point to anaphase onset as the consequence of a complex signaling cascade that occurs during mid- to late metaphase and appears to involve changes in cytosolic calcium and sequential changes in protein kinase and protein phosphatase activities. Our pharmacological studies have linked polyphosphoinositide cycling with this regulatory cascade (Wolniak, 1987; Larsen et al., 1989, 1991; Larsen and Wolniak, 1990) and have demonstrated the existence of a major regulatory transition point during mitosis that occurs midway through metaphase, ~15 min after NEBD. We found that the brief treatment of stamen hair cells with the protein kinase activator 1,2-dioctanoylglycerol (but not 1,3-dioctanoylglycerol) at different times during prometaphase or metaphase altered metaphase transit time in a way that suggests that protein kinase activity predominates in prometaphase and early metaphase and again late in metaphase (Larsen and Wolniak, 1990). Brief treatments of stamen hair cells with protein phosphatase inhibitors at different times during prometaphase and metaphase demonstrated that an important part of the cascade that culminates in anaphase is the activation of one or more protein phosphatases during mid-metaphase (Wolniak and Larsen, 1992).

Protein phosphatase activation also appears necessary for the synchronous separation of sister chromatids (Wolniak and Larsen, 1992), but asynchronous entry into anaphase does not appear to involve either major changes in spindle organization or the disruption of attachments between kinetochores and spindle microtubules (Larsen and Wolniak, 1993). The transition from elevated kinase to elevated phosphatase activity occurs ~15 min after NEBD. In untreated cells, we regularly observe an episode of chromatid unwinding at this time point, in which the sister chromatids separate from each other at areas distal to their centromeric regions in a fashion similar to that of an unraveling piece of rope. We infer that this unwinding process may be a morphological manifestation of a major physiological transition that occurs midway through metaphase (Wolniak, 1987, 1988; Larsen et al., 1991; Wolniak and Larsen, 1992).

From our previous work, it appears that an increase in protein phosphatase activity separates two intervals of elevated protein kinase activity during metaphase in these cells (Wolniak and Larsen, 1992), but the logical test for this interpretation would be to treat them briefly at known time points during prometaphase and metaphase with protein kinase inhibitors. Thus, the current experiments were designed to test the hypothesis that protein kinase activity predominates in prometaphase to early metaphase and again later in mid- to late metaphase in stamen hair cells. In the first part of this study, we describe a series of perfusion experiments in which cells were treated at known time points during prometaphase and metaphase with the broad spectrum protein kinase inhibitors K-252a, staurosporine, or calphostin C. Then, we describe a series of microinjection experiments in which stamen hair cells were impaled and loaded with known quantities of a nine–amino acid protein kinase substrate peptide.

All of the protein kinase inhibitors used in the current perfusion experiments were originally used to block protein kinase C (PKC) activity in animal cells (Tamaoki et al., 1986; Davis et al., 1989; Herbert et al., 1990). Although PKC is not among the major protein kinases in plants (Harmon, 1990; however, see Elliott and Kokke, 1987; Morello et al., 1993), K-252a blocks the activity of several plant protein kinases (Grosskopf et al., 1990). Both K-252a and staurosporine appear to inhibit the calcium-dependent protein kinase known as CDPK (Harmon and McCurdy, 1990), an abundant protein kinase that so far is found only in plants and protists (Harmon et al., 1987; Harmon and McCurdy, 1990; Harper et al., 1991; Roberts and Harmon, 1992; Schaller et al., 1992) and may be involved in calcium-dependent signaling cascades. Both K-252a and staurosporine could affect a variety of other plant protein kinases (Roberts and Harmon, 1992, review; Martiny-Baron and Scherer, 1989; Polya et al., 1989; Verhey et al., 1993; Watillon et al., 1993). Calphostin C is more specific than K-252a or staurosporine for PKC inhibition (Ohmi et al., 1990), but all of these drugs may act on other enzymes in distinct signaling cascades (Svetlov and Nigam, 1993); to our knowledge, calphostin C is not known to affect plant protein kinases.

Inasmuch as K-252a and staurosporine have been shown to block mammalian cell proliferation (Ohmi et al., 1990; Minana et al., 1993), and because staurosporine has recently been shown to be an inhibitor of $p34^{cdc2}$ protein kinase (Kitagawa et al., 1993), an enzyme known to exist in plants (for example, Feiler and Jacobs, 1990; Colasanti et al., 1991; Ferreira et al., 1991; Hirt et al., 1991; Hasimoto et al., 1993; John et al., 1993), we reasoned that these broad-spectrum inhibitors may be suitable for use on plant cells in which their specific efficacies have yet to be determined (however, see Grosskopf et al., 1990). We also reasoned that these drugs would serve as suitable general-purpose kinase inhibitors in experiments designed to demonstrate when, during mitosis, yet-to-be identified protein kinases are activated. Through empirical testing, we found minimal, nonlethal concentrations of K-252a and staurosporine that would induce changes in metaphase transit times as a function of their time of addition to the cells. We found maximal transit times observed with treatments initiated early or late in metaphase and minimal transit times with treatments initiated at 15 to 21 min after NEBD.

Our perfusion experiments (Larsen and Wolniak, 1990; Wolniak and Larsen, 1992) provide a general sense of how the cascade involves both protein kinases and phosphatases that act in a serial fashion to regulate progression into anaphase. In an effort to define the precise timing of protein kinase activation steps as well as the number of kinase elevation events occurring during prometaphase and metaphase, stamen hair cells were microinjected at known time points during prometaphase and metaphase with the nine–amino acid

PKC substrate peptide VRKRTLRRL (House and Kemp, 1987; Soderling, 1993). In the course of designing these experiments, we reasoned that VRKRTLRRL should serve as a suitable substrate for a variety of protein kinases, several of which are likely candidates in the regulatory cascade that mediates mitotic progression. If used properly, this general-purpose substrate peptide would provide insight into the timing and complexity of the cascade and serve as a foundation for later determinations of kinase identities. At high concentrations, we found that this peptide acts as a general inhibitor to mitotic progression, but far more importantly, the microinjection of very low levels of the peptide into the cytosol results in metaphase transit times that are altered in cells as a function of the specific time during metaphase when the peptide was introduced into the cytosol. We found five intervals during prometaphase and metaphase when these microinjections result in significantly extended metaphase transit times. These brief intervals are separated by periods in metaphase when identical microinjections are followed by normal times of entry into anaphase. These brief intervals for injection that result in extended metaphase transit times appear to be inflection points when protein kinase activity is being initiated or nearing completion in a complex, multistepped regulatory cascade that culminates in sister chromatid separation at anaphase onset.

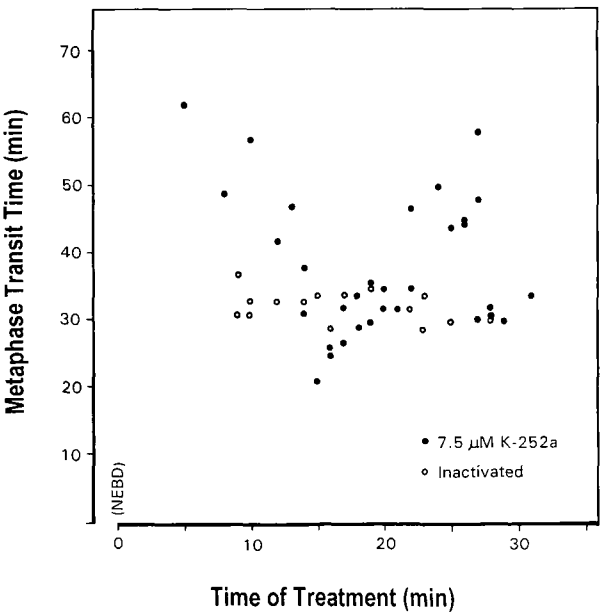

Figure 1. Metaphase Transit Time, the Interval from NEBD to Anaphase Onset, of *Tradescantia* Stamen Hair Cells Plotted as a Function of the Start Time of Treatment with 7.5 μM K252a (●) or 7.5 μM Microwave-Inactivated K-252a (○).

Each point on the plot represents the response of a separate cell. All treatments were administered by perfusion, and all of the treatments lasted 10 min. Inhibitor treatments initiated in prometaphase/early metaphase or late in metaphase resulted in delayed entry into anaphase. Inhibitor treatments initiated in mid-metaphase resulted in normal metaphase transit times. Metaphase transit times for normal cells are 33 min ± 2 min (SD).

RESULTS

Perfusions of Cells with the Protein Kinase Inhibitors K-252a, Staurosporine, and Calphostin C

We treated stamen hair cells by perfusion for brief intervals with the permeant protein kinase inhibitors K-252a, staurosporine, or calphostin C at known times during prophase, prometaphase, and metaphase and monitored subsequent rates of mitotic progression. K-252 or staurosporine perfusions initiated in early prometaphase or late metaphase each resulted in a significant delay in anaphase onset, whereas treatments initiated in mid-metaphase resulted in normal metaphase transit times. The metaphase transit times observed with perfusions of K-252a and staurosporine are presented in Figures 1 and 2, respectively. Our 10-min treatments with either 10 μM K-252a (Figure 1; Table 1) or 7.5 μM staurosporine (Figure 2; Table 1) affect the metaphase transit time primarily as a function of the time of initial exposure to the drug, whereas 10-min exposures to calphostin C had no effect on metaphase transit times in these cells (Table 1).

There are obvious temporal similarities in the responses of the cells to exposures to K-252a and staurosporine (Figures 1 and 2) that are presented together graphically in Figure 3 and compared statistically in Table 1. The minimal metaphase transit times were obtained with K-252a; these were in the 21- to 28-min range and followed perfusions initiated 15 to 17 min after NEBD (Figure 1). Similarly, the minimal metaphase transit times obtained with staurosporine, which were in the 26- to 30-min range, followed perfusions that were initiated 14 to 19 min after NEBD (Table 1; Figure 2). For treatments with K-252a or staurosporine that resulted in forestalled entry into anaphase, there were subtle temporal differences in the responses exhibited by the cells (Figure 3): there were differences both in the times of treatment eliciting maximally extended metaphase transit times and in the extent of delayed entry into anaphase. Maximal metaphase transit times that exceeded 40 min were observed in cells treated with K-252a <12 min after NEBD or 22 to 27 min after NEBD (Figure 1). The peak in metaphase transit times for K-252a treatments administered in late metaphase occurred at ~25 min after NEBD (Figures 1 and 3). Metaphase transit times approaching or exceeding 40 min were observed in cells treated with staurosporine 11 to 14 min after NEBD and again 25 to 27 min after NEBD (Figures 2 and 3). These extensions in the metaphase transit times are statistically significant when compared with metaphase transit times observed in control-treated, untreated, or calphostin C–treated cells (Table 1). The extensions of metaphase transit times for K-252a treatments administered in mid- to late metaphase (Figures 1 and 3) were greater than those observed with staurosporine (Figures 2 and 3).

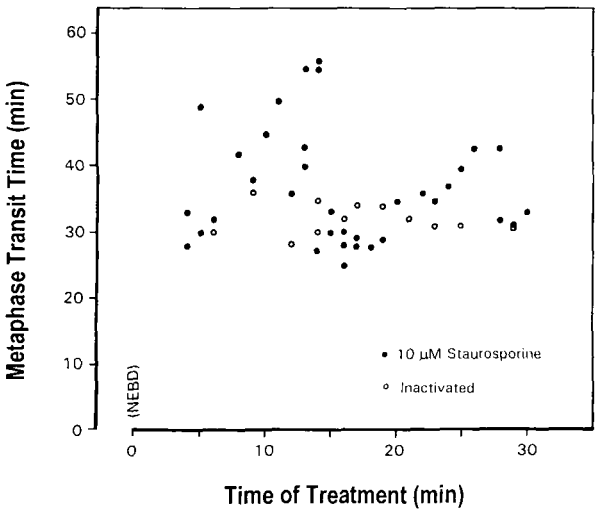

Figure 2. Metaphase Transit Time of Stamen Hair Cells Plotted as a Function of the Start Time of Treatment with 10 μM Staurosporine (●) or 10 μM Microwave-Inactivated Staurosporine (○).

Each point on the plot represents the response of a separate cell. All treatments were administered by perfusion, and all of the treatments lasted 10 min. Inhibitor treatments initiated in prometaphase/early metaphase or late in metaphase resulted in delayed entry into anaphase. Inhibitor treatments initiated in mid-metaphase resulted in normal metaphase transit times. Metaphase transit times for normal cells are 33 min ± 2 min (SD).

In stamen hair cells, we define the duration of anaphase as the interval from anaphase onset to cell plate vesicle aggregation (Table 1). Typically, cell plate vesicle aggregation occurs 19 min ± 1 min after anaphase onset in untreated cells. The aggregation of cell plate vesicles appears morphologically in optical section with differential interference contrast microscopy as a line transecting the cell at, or nearly at, a plane perpendicular to its longitudinal axis. In cells treated with K-252a, staurosporine, or calphostin C, we observed greater variability in the timing of cell plate vesicle aggregation than that observed in untreated cells (Table 1). Rates of anaphase chromosome separation appeared to be within the normal range of ~1.5 μm/min. Although the cells had been treated briefly with the inhibitors and the inhibitor had been removed as much as 45 min prior to cell plate vesicle aggregation, it remains unclear why cell plate formation almost always occurred at an oblique axis in the filament. The underlying mechanism for anomalous oblique plate formation is under study in our laboratory.

None of our control perfusions with microwave-inactivated K-252a (Figure 1; Table 1), microwave-inactivated straurosporine (Figure 2; Table 1), or microwave-inactivated calphostin C (Table 1) resulted in significant changes in the metaphase transit times observed. As shown in composite form in Figure 3, the changes in metaphase transit times observed with K-252a or staurosporine are complementary to changes in metaphase transit times that we observed when cells were treated at various times during metaphase with 1,2-dioctanoylglycerol (a lipid-dependent protein kinase activator; Larsen and Wolniak, 1990) or with okadaic acid, microcystin-LR, or microcystin-RR (protein phosphatase inhibitors; Wolniak and Larsen, 1992). Combined, these results suggest that one or more protein kinase activities are elevated in prometaphase/early metaphase and again later in metaphase (Figure 3).

Microinjection of VRKRTLRRL, a Protein Kinase Substrate Peptide, into Mitotic Stamen Hair Cells

To define more precisely the timing of protein kinase activation steps, we microinjected known quantities of the nine–amino acid protein kinase substrate peptide VRKRTLRRL into living stamen hair cells at known times during prophase, prometaphase, and metaphase. At concentrations approaching 1 mM in the cytosol, the microinjection of VRKRTLRRL at different times during prometaphase or metaphase lengthened the metaphase transit times of stamen hair cells and generally served as an inhibitor to progression through metaphase (data not shown). The overall pattern of delayed entry into anaphase after these injections with high levels of the peptide resembled the effects of K-252a (Figure 1) or staurosporine (Figure 2), with shortest metaphase transit times resulting from injections performed in mid-metaphase. However, even for the injections with 1 mM VRKRTLRRL performed 15 to 22 min after NEBD, the transit times were significantly longer than normal (data not shown). As a consequence, the experimental approach was modified, and the final cytosolic concentration of the peptide was lowered significantly to the 100 nM to 1 μM range.

At this low concentration in the cytosol, we reasoned that this substrate peptide should act as a competitive inhibitor only in limited time frames either when its concentration approached that of endogenous substrates or when protein kinase activity is submaximal. That is, VRKRTLRRL should provide an indication of inflection points at which protein kinases are nearly inactivated and endogenous substrate phosphorylation is nearing completion. By the same logic, we reasoned that at other times during metaphase, when protein kinases participating in the cascade are maximally active or when endogenous substrate levels are high, the addition of a small quantity of the VRKRTLRRL substrate should not alter the rate of mitotic progression in these cells. We reasoned further that VRKRTLRRL may slow progression at time points when protein kinases are just becoming active, under conditions in which the kinases themselves are part of the substrate pool for phosphorylation. In this last scenario, VRKRTLRRL could reduce the effective number of protein kinase molecules acting on available substrates in the cascade. The cytosolic concentration range of the peptide inducing a time-dependent response was determined empirically in several series of microinjection experiments (data not shown).

With this rationale as the basis for our approach, VRKRTLRRL was microinjected into cells at a final cytosolic

concentration of ~100 nM to 1 μM at known time points after NEBD. The resultant metaphase transit times are depicted as a function of the time of microinjection in Figure 4. As in Figures 1 and 2, each data point on this plot represents the response from one individual cell. Statistically significant changes in the metaphase transit time were defined primarily by the time that the peptide was introduced into the cell (Figure 4; Table 2). The metaphase transit times observed after the injection of 100 nM to 1 μM VRKRTLRRL at various times during prometaphase and metaphase (Figure 4) followed a series of "spikes and valleys," which are depicted graphically in Figure 5 and compared statistically in Table 2. Four of our microinjections of VRKRTLRRL, performed <4 min after NEBD, resulted in extended metaphase transit times (Figure 4; Table 2). In addition, there are four additional specific intervals during prometaphase and metaphase, some of which last only 1 to 2 min, during which microinjections of low levels of VRKRTLRRL resulted in statistically significant increases of the metaphase transit time. Microinjections performed at ~8 min, 14 to 16 min, 21 min, and 24 min after NEBD all resulted in significant extensions in the metaphase transit times (Figures 4 and 5; Table 2). In contrast, microinjections performed 4 to 7 min, 10 to 13 min, 17 to 20 min, and 22 to 23 min after NEBD all resulted in the normal or somewhat shortened metaphase transit times observed (Figures 4 and 5; Table 2).

In control experiments, the microinjection of microwave-inactivated VRKRTLRRL resulted in no significant changes in the timing of anaphase onset from those observed with uninjected cells (Figure 4; Table 2). In other controls, microinjections of carrier buffer alone did not significantly alter the metaphase transit times of these cells (Table 2), unless the cell was impaled with excessive force (Wolniak and Larsen, 1992). In several different experiments, the intentional use of a high force for impalement was followed by the injection of carrier buffer alone, carrier buffer with VRKRTLRRL, or carrier buffer with microwave-inactivated VRKRTLRRL. These cells all entered anaphase ≤33 min after NEBD, irrespective of the time of impalement (data not shown). Thus, for reasons described in Methods, if the impalement force in a given experiment was excessive, that cell was not included in the data set. In a separate set of control injections, the microinjection of 100 nM to 1 μM VRKRALRRL, a similar but nonphosphorylatable peptide,

Table 1. Metaphase Transit Times and Anaphase Durations for Stamen Hair Cells Treated Briefly by Perfusion with K-252a, Staurosporine, or Calphostin C during Prometaphase or Metaphase

Treatment	n[a]	Time of Addition after NEBD (min)	Metaphase Transit Time (min)	Significance	Anaphase Duration (min)
K-252a					
	7	5 to 14	46.6 ± 10.7	*[b]	21.6 ± 4.0
	12	15 to 21	30.6 ± 4.1	NS[c]	20.4 ± 3.0
	9	22 to 27	44.7 ± 8.2	*	20.6 ± 2.4
	4	28 to 31	32.1 ± 1.9	NS	18.0 ± 0.8
Control	15	9 to 28	32.1 ± 2.4	-	19.9 ± 3.5
Staurosporine					
	16	4 to 14	41.1 ± 9.6	*	23.4 ± 4.5
	10	15 to 21	29.5 ± 2.8	NS	21.4 ± 1.8
	7	22 to 28	38.0 ± 1.4	*	22.4 ± 2.0
	2	29 to 30	32.0 ± 1.4	NS	19.0 ± 0
Control	12	6 to 29	32.3 ± 2.8	-	18.3 ± 1.5
Calphostin C					
	5	5 to 14	32.8 ± 1.3	NS	20.6 ± 4.3
	6	15 to 21	31.0 ± 2.2	NS	21.8 ± 1.2
	8	22 to 30	31.6 ± 0.9	NS	21.5 ± 3.6
	19	5 to 30	31.7 ± 1.6	NS	21.4 ± 3.1
Control	7	2 to 27	32.7 ± 2.6	-	19.1 ± 1.5

Cells were treated by perfusion with one of the three protein kinase inhibitors and incubated in the inhibitor solution for 10 min before perfusion of carrier buffer lacking the drug. The cells exhibited a time or treatment dependence to K-252a or staurosporine in which metaphase transit times were extended significantly with treatments initiated early in prometaphase/metaphase or again late in metaphase. In contrast, calphostin C was without effect on the metaphase transit times in these cells. After treatments during prometaphase or metaphase with any of these inhibitors, the duration of anaphase varied to a greater extent than that observed in untreated cells. The control solutions consisted of perfusions with microwave-inactivated inhibitors, as described by Wolniak and Larsen (1992). The statistical significance of differences in metaphase transit times was determined by Student's *t* tests, as described by Wolniak and Larsen (1992).

[a] *n*, number.

[b] *, $P < 0.005$.

[c] NS, not significantly different.

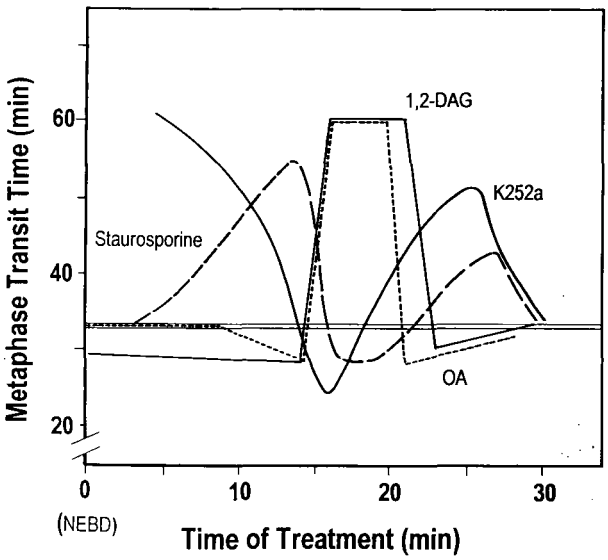

Figure 3. Progression toward Anaphase Involves Multiple Increases in Protein Kinase Activities and an Elevation of Protein Phosphatase Activity.

In this composite graph, metaphase transit times for populations of similarly treated cells are plotted as a function of the initial time of treatment with the perfusion solution. Perfusions in each kind of inhibitor experiment lasted 10 min before vigorous washout with carrier buffer. The staurosporine and K-252a response curves are derived from data presented in Figures 1 and 2. These curves are combined with the responses of cells to the addition of 1,2-dioctanoylglycerol (1,2-DAG), a protein kinase activator (Larsen and Wolniak, 1990), or to the addition of okadaic acid (OA), a protein phosphatase inhibitor (Wolniak and Larsen, 1992), to cells for 10-min intervals beginning at various times during prometaphase and metaphase. Identical perfusion treatments with microcystin-LR or microcystin-RR resemble the response of cells to okadaic acid (Wolniak and Larsen, 1992). The responses of the cells to the kinase and phosphatase inhibitors, the kinase activator, and the substrate peptide indicate that both protein kinase(s) and protein phosphatase(s) participate in the cascade that culminates in anaphase. These results suggest that at least two periods of elevated protein kinase activity, early and late in the interval from NEBD to anaphase onset, are punctuated by an interval of elevated protein phosphatase activity in mid-metaphase 15 to 22 min after NEBD. In prometaphase, in early metaphase, and again late in metaphase, protein kinase activity appears to predominate in the cascade that triggers anaphase. Moreover, protein kinase activities appear to be less important, or possibly suppressed, in mid-metaphase, a time when protein phosphatase activity is elevated.

resulted in significantly delayed entry into anaphase, irrespective of the time of its introduction into the cytosol. The mean metaphase transit time observed after microinjection with VRKTALRRL in prometaphase, early metaphase, mid-metaphase, and late metaphase was in excess of 48 min (Table 2).

VRKRTLRRL Acts in a Physiologically Relevant Way To Slow Mitotic Progression

The types of experiments we have performed on living cells do not provide any direct evidence that VRKRTLRRL is phosphorylated by endogenous protein kinases or even that the peptide is acting on mitotic progression through the inhibition of normally occurring regulatory events that precede anaphase onset. If VRKRTLRRL is acting through physiologically relevant competitive inhibition of protein kinase activity, then its presence in a cell should have predictable effects on events that are already known to be the consequence of protein phosphorylation. NEBD is such an event (Gerace and Blobel, 1980;

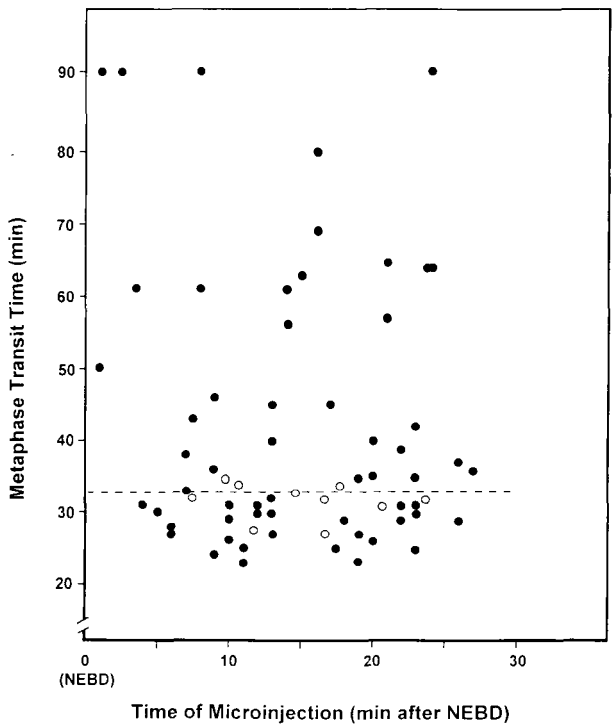

Figure 4. VRKRTLRRL, a Protein Kinase Substrate Peptide, Alters Metaphase Transit Times in Stamen Hair Cells as a Function of the Time of Its Microinjection into the Cytosol.

Each point on the plot represents the metaphase transit time of an individual cell plotted as a function of the time of microinjection with VRKRTLRRL (●). The peptide is present in the cytosol at a concentration of ~100 nM to 1 μM. Metaphase transit times are extended significantly after injections performed within 4 min of NEBD and thereafter in prometaphase and metaphase at four discrete intervals, at 8, 15, 21, and 24 min after NEBD. These peaks in metaphase times are separated by intervals when VRKRTLRRL injections result in normal metaphase transit times. Metaphase transit times for cells microinjected with microwave-inactivated VRKRTLRRL (○) are also depicted on this plot; the microwave-treated VRKRTLRRL had no effect on the metaphase transit times, irrespective of the time of its injection into a cell.

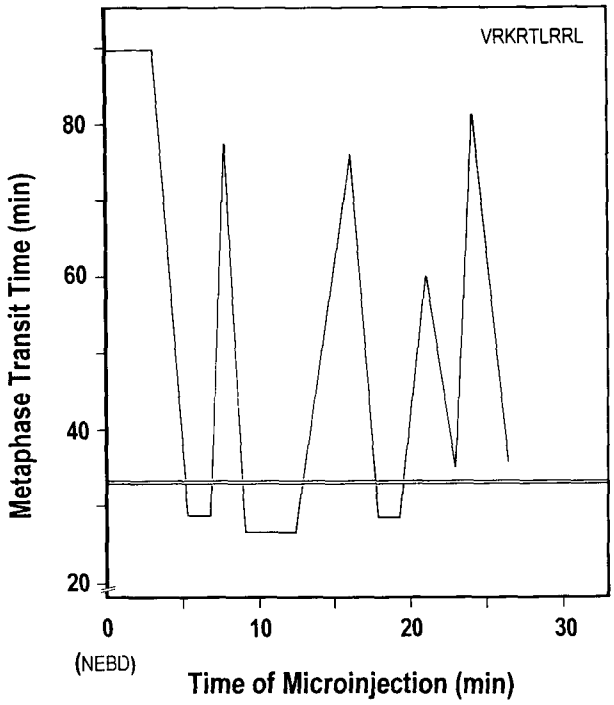

Figure 5. *Response of Cells to the Microinjection of VRKRTLRRL at Various Points during Prometaphase and Metaphase.*

A plot of metaphase transit times showing the response of cells to the microinjection of 100 nM to 1 μM VRKRTLRRL, derived from data points presented in Figure 4. The metaphase transit times observed for normal cells are 33 min ± 2 min (SD), depicted as the doubled horizontal lines on the graph. The microinjection of 100 nM to 1 μM VRKRTLRRL results in a series of peaks and valleys, when cells injected at 0 to 4, 8, 15, 21, and 24 min after NEBD exhibit significant increases in their metaphase transit times. In contrast, cells injected at 5 to 7, 10 to 13, 17 to 20, 22 to 23, and 26 to 27 min after NEBD exhibit essentially normal metaphase transit times. The peak at 15 min after NEBD coincides with the minima in the response curves observed after treatments with the kinase inhibitors depicted in Figure 3; this is a likely interval when both protein kinase activity and endogenous substrate levels are low and when the addition of a small amount of substrate could greatly affect the rate of progression through metaphase. The peaks at 8, 21, and 24 min after NEBD coincide with the early stages of the increases in the response curves observed after treatments with the protein kinase inhibitors K-252a and staurosporine and appear to represent (what we intrepret as early) periods of protein kinase activation. At these time points, we believe that the protein kinases affected by drug treatment are operating at submaximal levels. The presence of VRKRTLRRL introduced at different times during prometaphase and metaphase highlights inflection points in the regulatory cascade that precedes entry into anaphase. The two peaks observed with VRKRTLRRL late in metaphase suggest that multiple protein kinases become sequentially activated at specific times before anaphase onset. When combined with the information presented in Figure 3, the data indicate that the pattern of activity appears to be kinase–phosphatase–kinase or kinase–phosphatase–kinase–kinase, as cells progress through prometaphase, early/mid-metaphase, and then mid-/late metaphase.

Gerace and Burke, 1988; Heald and McKeon, 1990). When compared with the timing of anaphase onset, our ability to predict NEBD is less precise. Although we are not able to assess the timing of NEBD a priori in living stamen hair cells, we know that it follows the rapid process of spindle expansion by 3 to 5 min. Spindle expansion reaches its maximal extent as the nucleus becomes elliptical in optical section, and then the nuclear surface becomes crenulated in a process we have called nuclear compression (Wolniak and Larsen, 1992). Nuclear compression ends as the chromatin mass suddenly expands in area, as viewed in optical section; this relaxation event reports the occurrence of NEBD. These morphological markers make it possible to predict that NEBD will occur in late prophase cells within a few minutes. Occasionally, adjacent cells in a stamen hair will be dividing, and they are usually in different stages of mitosis. The metaphase transit time of one cell can serve as a control for microinjection and provide a time base for NEBD in the other cell. Stamen hair cells are coupled together through plasmodesmata, where the intercellular movement of ions and small molecules occurs rapidly (see Wolniak and Larsen, 1992). The movement of the injected substance itself or the movement of signaling ions or molecules affected by the injected substance can alter the timing of mitotic transitions in uninjected cells.

Figure 6 depicts the passage of two adjacent stamen hair cells through prometaphase, metaphase, and anaphase at different times. The proximal cell in the filament was ~26 min ahead of the distal cell in the filament at the start of the experiment. NEBD, visualized in real time by the "relaxation" of the chromosomal mass, is easily seen in the proximal cell of this pair (Figures 6A and 6B). At 23 min after NEBD, the proximal cell was impaled and microinjected with the VRKRTLRRL peptide (Figure 6E). The injected cell remained in metaphase for an additional 12 min, until the sister chromatids uncoiled a final time and split apart (Figure 6K), 35 min after NEBD. The injected cell progressed through anaphase normally and underwent cell plate vesicle aggregation 19 min after anaphase onset (Figure 6N). As we observed with perfusions of K-252a and staurosporine, the positioning of the plate was oblique relative to the longitudinal axis of the cell: the occurrence, extent, and explanation of this anomaly will be presented in detail elsewhere (P.M. Larsen and S.M. Wolniak, manuscript in preparation).

At the time of proximal cell injection, the distal cell was still in prophase (Figure 6E) but had begun to undergo nuclear compression, during which the nucleus became elliptical in optical section, presumably as a consequence of rapid spindle microtubule growth. The extent of compression in the uninjected (distal) cell should have been comparable to the compression observed in the proximal cell (Figures 6A and 6B) before it had been injected. However, successive images (Figures 6F to 6J) demonstrate that the nuclear envelope failed to break down normally in the uninjected cell. Instead, nuclear compression continued to occur in the uninjected cell without relaxation of the chromosomal mass until the nucleus in this

Table 2. Effect of Microinjections of the Kinase Substrate Peptide VRKRTLRRL at Various Times during Prometaphase and Metaphase on the Metaphase Transit Times of Stamen Hair Cells

Peptide/Solution	n[a]	Time of Injection after NEBD (min)	Metaphase Transit Time (min)	Significance
VRKRTLRRL	4	0 to 3.5	73 ± 20	*[b]
VRKRTLRRL	5	4 to 7	32 ± 4	NS[c]
VRKRTLRRL	5	7.5 to 9	47 ± 23	*
VRKRTLRRL	10	10 to 13	29 ± 5	NS
VRKRTLRRL	7	14 to 16	58 ± 16	*
VRKRTLRRL	10	17 to 20	32 ± 7	NS
VRKRTLRRL	2	21	61 ± 6	*
VRKRTLRRL	6	22 to 23	35 ± 5	NS
VRKRTLRRL	3	24	73 ± 15	*
VRKRTLRRL	3	≥25	34 ± 4	NS
Microwave-inactivated VRKRTLRRL	10	7 to 24	31 ± 3	–
Carrier buffer	7	5 to 25	32 ± 4	–
VRKRALRRL	5	8 to 17	>48	*

Stamen hair cells were microinjected at known time points during prometaphase and metaphase with the protein kinase substrate peptide VRKRTLRRL, with its nonphosphorylatable analog VRKRALRRL, or with carrier buffer, as described in the text. The final cytosolic concentration for each peptide was 100 nM to 1 μM. The time points were based on the occurrence on NEBD, an event defined as time = 0. Metaphase transit times were analyzed by time-lapse video microscopy; the normal metaphase transit time in this cell type is 33 min ± 2 min. During specific, brief intervals, the metaphase transit times were lengthened significantly (Student's t test; *, $P < 0.005$), whereas for other cells injected a few minutes earlier or a few minutes later, metaphase transit times were not significantly (NS) different from controls. We saw no significant changes in the metaphase transit times in cells microinjected with either microwave-inactivated VRKRTLRRL or carrier buffer from untreated cells, irrespective of the time of impalement. In contrast, the microinjection of VRKRALRRL, a nonphosphorylatable analog of VRKRTLRRL, resulted in extended metaphase transit times after injections performed at a number of points during prometaphase and metaphase. A precise mean for the metaphase transit time obtained in cells injected with VRKRALRRL was not determined, because in one of the five cells counted in this sample, the cell remained in metaphase 80 min after NEBD, when the experiment was terminated.

[a] n, number.

[b] *, $P < 0.005$.

[c] NS, not significantly different.

cell was nearly flattened in optical section, relative to the longitudinal axis of the mitotic apparatus (Figure 6J). We estimate that NEBD was delayed in the uninjected cell by ~8 to 10 min. Chromosomal relaxation (that is, NEBD) in the uninjected cell occurred in synchrony with anaphase onset in the adjacent, microinjected cell (Figure 6K).

It is possible that VRKRTLRRL moved from the injected cell into the uninjected cell through plasmodesmata. If the VRKRTLRRL peptide is acting as a phosphorylatable substrate in both of these cells and its concentration is sufficiently low so that it interferes effectively with phosphorylation only when its concentration approaches endogenous substrate levels or when protein kinases are operating at submaximal rates, then it is reasonable to expect that the metaphase transit time for the uninjected cells will be normal. This is exactly what we observed: anaphase onset in the uninjected cell occurred at 33 min after NEBD (Figures 6O and 6P). As in the injected cell, the interval from anaphase onset to cell plate vesicle aggregation was normal at 19 min (Figure 6R). Alternatively, VRKRTLRRL present in the injected cell may affect levels of cytosolic calcium or polyphosphoinositide levels as an indirect consequence of protein kinase inhibition, and these changes are manifested in the adjacent, uninjected cell as a delay in NEBD.

DISCUSSION

In this study, we show that the brief treatment of stamen hair cells during prometaphase or metaphase with the protein kinase inhibitors K-252a or staurosporine results in changes in the metaphase transit times that are dependent on the initial time of treatment with the drug. These shifts in the metaphase transit times are maximal after inhibitor treatments administered early or late in metaphase and minimal after treatments administered in mid-metaphase. Our results suggest that protein kinase activity necessary for entry into anaphase is elevated during these intervals. In contrast, we observed no change in metaphase transit times in cells treated during prometaphase or metaphase with the protein kinase inhibitor calphostin C. We suspect that this difference in response results from the narrow specificity of calphostin C for PKC, an enzyme that appears not to be abundant in higher plants (Harmon, 1990), as opposed to the somewhat broader specificity of staurosporine and K-252 for calmodulin-domain protein kinases, some of which are known to exist in plants (Roberts and Harmon, 1992, review).

We believe that K-252a and staurosporine affect metaphase transit times through the inhibition of one or more protein kinases whose activities are required for progression through

metaphase and entry into anaphase, and we interpret our results as evidence that entry into anaphase is dependent on several successive intervals during which protein kinase activity is elevated (Figure 3). Protein kinase elevations early in prometaphase/early metaphase and again late in metaphase are separated by an interval of elevated protein phosphatase activity in mid-metaphase. The elevation of protein phosphatase activity is necessary for the synchronous separation of sister chromatids at anaphase onset in both plant and animal cells (Wolniak and Larsen, 1992; Larsen and Wolniak, 1993) but does not appear to be involved in gross aspects of spindle organization or in the attachment of spindle microtubules to the kinetochores (Larsen and Wolniak, 1993).

Although we are not able to identify the specific protein kinases affected by these inhibitors in the individual plant cells that we study, it seems clear that their mode of activity in the timing of mitotic events involves protein kinases, because the changes in metaphase transit times observed with K-252a (Figures 1 and 3) and staurosporine (Figures 2 and 3) are complementary to changes we observed after treatments of cells with 1,2-dioctanoylglycerol (an activator of lipid-dependent protein kinases; Larsen and Wolniak, 1990; Figure 3). Treatments with 1,2-dioctanoylglycerol administered in mid-metaphase generate metaphase transit times in excess of 55 min, and precocious entry into anaphase followed treatments administered early or late in metaphase (Larsen and Wolniak, 1990; Figure 3). In that study (Larsen and Wolniak, 1990), we hypothesized that 1,2-dioctanoylglycerol activated a protein kinase that phosphorylated a variety of substrates, some of which had to be phosphorylated for entry into anaphase. Delayed progression into anaphase was the result of either the phosphorylation of substrates that cannot be phosphorylated before anaphase onset or the mid-metaphase activation of one or more protein phosphatases. We tested the latter idea by treating cells briefly during metaphase with okadaic acid, microcystin-LR, or microcystin-RR, all potent protein phosphatase inhibitors (Wolniak and Larsen, 1992). These treatments resulted in a response that closely resembled the result obtained with 1,2 dioctanoylglycerol (Larsen and Wolniak, 1990; Figure 3) in which metaphase transit times were doubled as a consequence of treatments initiated in mid-metaphase. Collectively, these results summarized in Figure 3 suggest that entry into anaphase involves multiple protein kinase activation steps early and late in metaphase and that these episodes are punctuated by an interval of elevated protein phosphatase activity in mid-metaphase (Wolniak and Larsen, 1992).

The microinjection of VRKRTLRRL into stamen hair cells at known times during prometaphase or metaphase resulted in a complex and time-dependent response (Figures 4 and 5) that, when superimposed on the response curves obtained with stamen hair cells after brief treatments with 1,2-dioctanoylglycerol, okadaic acid, K-252a, and staurosporine (Figure 3), indicates transitional periods during prometaphase and metaphase when changes in phosphorylation and dephosphorylation activities occur. These transitional periods occur at 0 to 4, 8, 15 to 16, 21, and 24 min after NEBD (Figure 5). The VRKRTLRRL microinjections were designed to provide the peptide at a final concentration of 100 nM to 1 μM in the cytosol. Under these conditions, the peptide should act as a competitive inhibitor of protein kinase activity under conditions when kinases are operating at a submaximal level and, additionally, under conditions when the level of VRKRTLRRL approaches the concentration of endogenous substrates whose phosphorylation is necessary for entry into anaphase. The spikes observed at 8, 21, and 24 min after NEBD (Figures 4 and 5) overlie the increases in metaphase transit times observed with treatments administered at comparable times with K-252a and staurosporine (Figure 3). We believe that these intervals in prometaphase and late metaphase are times when protein kinase activites are submaximal but increasing in the cell. These results provide an in vivo demonstration that a multifaceted, sequential cascade actually occurs during mitosis.

The spike in metaphase transit time observed with VRKRTLRRL microinjections performed 14 to 16 min after NEBD (Figure 5) coincides with the minimal transit times observed with K-252a and staurosporine (Figure 3); we believe that this time point represents a stage in mitosis when the phosphorylation of endogenous substrate is nearly completed, and the addition of $\sim 10^5$ to 10^6 copies of VRKRTLRRL slows progression through competitive inhibition. The injection of VRKRTLRRL at other times in metaphase (for example, 10 to 13 min after NEBD) does not slow progression into anaphase, because we think that the amount of peptide added to the cell is insignificant when compared with that of endogenous substrate. A possible explanation for the brief duration of the intervals during metaphase when the injection of VRKRTLRRL extends the metaphase transit time is that the substrate/peptide acts as a competitive inhibitor for the phosphorylation of regulatory enzymes themselves (for example, protein kinases and/or protein phosphatases), which would function, after (auto)phosphorylation, as active components of the cascade responsible for entry into anaphase. In this context, a rather small pool of VRKRTLRRL could act as an unusually effective inhibitor of mitotic progression at distinct, short, and specific time frames during metaphase.

Although we are unable to detect changes in the phosphorylation of specific peptides in individual cells in our current experiments, we have a clear indication that the placement of VRKRTLRRL into the cytosolic space of a mitotic stamen hair cell results in the alteration of events that should be linked to protein kinase activity. In Figure 6, we show how the microinjection of VRKRTLRRL into a metaphase cell affects NEBD in an adjacent cell still in prophase. It is reasonable to suspect that NEBD, manifested morphologically as relaxation of the chromosomal mass, is linked to the phosphorylation of nuclear lamins (Gerace and Blobel, 1980; Gerace and Burke, 1988; Heald and McKeon, 1990). We interpret the flattening of the nucleus in the uninjected pair of adjacent dividing cells (Figures 6F to 6J) as a consequence of delayed lamin phosphorylation. Delayed NEBD coupled with spindle expansion would

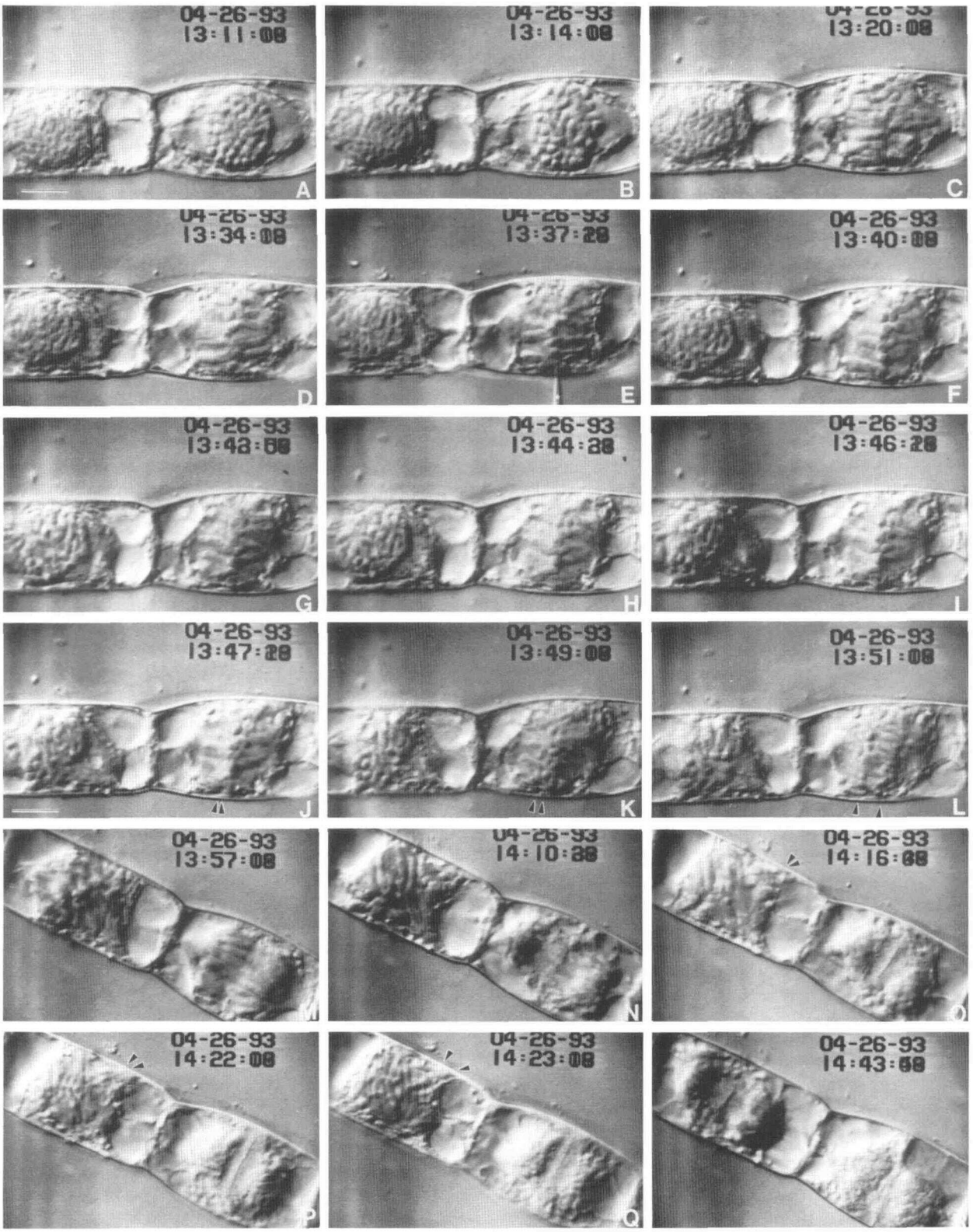

Figure 6. NEBD Is Delayed by the Microinjection of VRKRTLRRL into Stamen Hair Cells.

be displayed as a flattening of the chromosomal mass in the absence of the chromosomal relaxation event. It seems likely that lamin phosphorylation in vivo is an event that is inhibited by the presence of VRKRTLRRL, but an alternative explanation for the delay of NEBD in the uninjected cell is that VRKRTLRRL affects protein kinase activity in the injected cell and, consequently, exerts an indirect effect on the pools of signaling ions and molecules, such as calcium and polyphosphoinositides, respectively. The reason why NEBD in the uninjected cell accompanies anaphase onset in the injected cell is not entirely clear but is consistent with the notion of a signaling event that can be transmitted between cells (presumably through plasmodesmata). It is plausible that altered pools of signaling components that are readily transported between cells could account for delays in morphological transitions during mitosis, such as those observed in Figure 6. The identity of such a signal has not been determined but could be a transient rise in cytosolic calcium activity (Wolniak, 1988; Hepler, 1989, 1994).

The spikelike peaks in metaphase transit times observed after VRKRTLRRL injections at 8 and 15 min after NEBD are times in untreated cells when sister chromatid arms spontaneously unwind from each other and then, a few minutes later, wind back together. This cyclic process of uncoiling and recoiling is visible in real time and with replay of time-lapse videotapes but is difficult to depict in still micrographs, except right before anaphase onset (for example, Figures 6H, 6I, and 6J in the injected cell). Chromatid unwinding and rewinding may represent the morphological manifestation of significant shifts in physiological activity in the cytosolic compartment. The time point at 15 min after NEBD is when the cells become extremely sensitive to 1,2-dioctanoylglycerol (Larsen and Wolniak, 1990; Figure 3) or to okadaic acid (Wolniak and Larsen, 1992; Figure 3) treatments and appears to be a time in mid-metaphase of protein phosphatase activation that is necessary for entry into anaphase. The decline in sensitivity to okadaic acid at ~20 to 21 min after NEBD (Figure 3; Wolniak and Larsen, 1992) coincides with a period of increased sensitivity to both K-252a and staurosporine and with the increased metaphase transit times that follow the microinjection of VRKRTLRRL at 21 min after NEBD. We believe it is possible that our observed changes in metaphase transit times at this stage of metaphase are the result of a direct perturbation of components in the phosphorylation cascade. Recently, Dohadwala et al. (1994) demonstrated that $p34^{cdc2}$/cyclin-A or $p34^{cdc2}$/cyclin-B protein kinase could phosphorylate a specific threonine residue on several isoforms of protein phosphatase 1 and thereby induce inactivation of the phosphatase. They suggested that through this phosphorylation event, protein phosphatase activity could be regulated in vivo. We suspect that when administered at 20 to 22 min after NEBD, our kinase inhibitors and our kinase substrate peptide could increase metaphase transit times through this mechanism.

We were initially surprised that the nonphosphorylatable peptide VRKRALRRL slowed progression through metaphase, irrespective of the time of its injection into the cell (Table 2). However, the peptide exhibits amino acid identity at eight of nine residues and probably binds to the active site of a

Figure 6. (continued).

Differential interference contrast photomicrographs of a pair of adjacent stamen hair cells both undergoing mitosis.

(A) The proximal cell (right) is further along in prophase than the distal cell (left). Spindle expansion has begun in the proximal cell, and the nucleus is beginning to undergo compression.

(B) NEBD is shown in the proximal cell. The distal cell is beginning to build a spindle, which appears as clear zones, triangular in shape, at the surface of the nucleus.

(C) and **(D)** Metaphase in the proximal cell and prophase in the distal cell are shown.

(E) The proximal cell, in metaphase, is microinjected with VRKRTLRRL 23 min after NEBD. As described in the text, the final cytosolic concentration of VRKRTLRRL was ~100 nM. The distal cell is still in prophase and is still building a spindle. (The molecular weight of VRKRTLRRL is ~2100 and should be small enough to pass from cell to cell via plasmodesmata.)

(F) The proximal cell is in metaphase, and the distal cell is in prophase.

(G) to **(J)** The proximal cell is in metaphase and is undergoing cycles of sister chromatid uncoiling and recoiling. The distal cell is undergoing anomalous compression of the nucleus. The nucleus in this cell becomes increasingly flattened.

(K) Anaphase onset in the proximal cell is shown 35 min after NEBD. The distal cell has just undergone NEBD. Significantly, the nucleus in the distal cell underwent extensive flattening during an extended period of compression, as if lamin phosphorylation were delayed by the presence of the peptide in the cytosol at the time of NEBD.

(L) and **(M)** The proximal cell is in early anaphase, and the distal cell is in prometaphase. For images in **(M)** through **(R)**, the stage has been rotated around the optic axis of the microscope for greater clarity of chromosomal behavior.

(N) The proximal cell is in late anaphase and is about to begin cell plate vesicle aggregation, an event that defines the start of cytokinesis. The distal cell is in metaphase.

(O) Cell plate vesicle aggregation has occurred in the proximal cell, and sister chromatid uncoiling is occurring at the metaphase plate of the distal cell.

(P) The proximal cell is making a cell plate. Anaphase onset in the distal cell is shown 33 min after it exhibited NEBD. (The presence of VRKRTLRRL before NEBD had no effect on metaphase transit time.)

(Q) The proximal cell continues to make its cell plate. Anaphase is shown in the distal cell.

(R) The proximal cell continues to make its cell plate. Cell plate vesicle aggregation has occurred in the distal cell.

Bars in **(A)** and **(J)** = 10 μm.

protein kinase with essentially the same affinity as threonine-containing peptide. Because it cannot be phosphorylated and because it is reasonable to suspect that phosphorylation of a substrate would result in a loss of binding affinity for the active site of a protein kinase, it is reasonable to believe that VRKRALRRL is acting as a potent inhibitor for the kinase as opposed to a suitable substrate for the kinase. Of course, it is possible, albeit less likely, that VRKRALRRL slows progression through metaphase through some nonspecific inhibitory mechanism.

At this point, we are not able to address the obvious and important question of which particular protein kinase(s) is responsible for the phosphorylation of VRKRTLRRL and which protein kinase(s) is being inhibited by VRKRALRRL. However, it is clear that the threonine-bearing version of the peptide is a suitable substrate for PKC (House and Kemp, 1987; Kemp and Pearson, 1991), an enzyme that is probably rare in plant cells (Roberts and Harmon, 1992; MacKintosh and MacKintosh, 1994), and a reasonable substrate for CDPK (Roberts and Harmon, 1992). With its high arginine content, VRKRTLRRL may also serve as an acceptable substrate for $p34^{cdc2}$/cyclin (Shenoy et al., 1989; Peter et al., 1990) in spite of the absence of a proline near the phosphorylation site. Based on its primary sequence, VRKRTLRRL may also be a suitable substrate for some of the calcium/calmodulin-dependent protein kinase isozymes (Lee et al., 1994). Both $p34^{cdc2}$/cyclin and CDPK protein kinases are known to exist in plants, and both are likely, but certainly not the only, candidates for the regulatory cascade that regulates entry into anaphase. In cytoplasmic extracts of Xenopus, calcium/calmodulin-dependent protein kinase II has been shown to be involved indirectly in the induction of anaphase through its activation of a ubiquitin pathway that functions in cyclin degradation (Morin et al., 1994). Calcium/calmodulin-dependent protein kinases exist in plants (Roberts and Harmon, 1992). Our future efforts will be directed toward the identification of protein kinases involved in this mitotic regulatory cascade, in part through the microinjection of peptide substrates that exhibit high specificity for particular enzymes.

METHODS

Purified cutinase was kindly provided by Dr. P.E. Kolattukudy (Ohio State University, Columbus). K-252a, staurosporine, low-melt agarose, and VRKRTLRRL (fast protein liquid chromatography [FPLC]-purified) were obtained from Sigma (St. Louis, MO). Calphostin C was obtained from Calbiochem (La Jolla, CA). VRKRALRRL was synthesized and purified by FPLC at the Protein and Nucleic Acid Laboratory at the University of Maryland. Unless otherwise indicated, all other reagents were also obtained from Sigma.

Spiderwort plants, *Tradescantia virginiana* cv Zwanenburg blue, were maintained in the University of Maryland greenhouse facilities under an 18-hr photoperiod, as described previously by Wolniak and Larsen (1992). Stamen hairs were dissected from immature flower buds of spiderwort plants, as described previously by Wolniak and Bart (1985a) and Wolniak and Larsen (1992).

Perfusion Protocols

For our perfusion experiments, stamen filaments with attached hairs were incubated in cutinase at pH 8.0 for 40 min. After this incubation, the filaments were returned to 15 mM Hepes, 15 mM KCl, pH 7.0 to 7.2, for further dissection and microscopic observation. After locating an appropriate cell, the preparation was perfused with 20 to 50 μL of Hepes/KCl buffer containing 10 μM K-252a, 7.5 μM staurosporine, or 10 μM calphostin C. The perfusions were performed at predetermined times after nuclear envelope breakdown (NEBD), an event that served as time = 0 for all of our experiments. NEBD in these cells is readily discernible in real time (Wolniak and Bart, 1985a; Wolniak and Larsen, 1992) but can be defined with certainty through videotape replay. All of our treatments are expressed as a function of the time of NEBD. For these perfusion treatments, the Hepes/KCl medium beneath the coverglass was completely replaced within ~20 sec. Flow of the perfusion solution beneath the coverglass was sustained by the placement of a Kimwipe wick (Kimberly-Clark, Atlanta, GA) at one edge of the coverglass after the placement of ~50 μL of solution at the opposite edge of the coverglass. A total of at least 100 μL of solution was passed beneath the coverglass for each treatment. Small dabs of Vaseline, present at each corner of the coverglass, helped to keep the distance between the coverglass and the slide approximately constant. In this kind of preparation, the cells remained stationary, intact, and healthy both during and after the rapid exchange of solutions beneath the coverglass. Thus, the cells were rapidly exposed to a constant concentration of inhibitor. The cells were incubated for 10 min with the drug before the rapid-flow perfusion of excess Hepes/KCl buffer lacking the drug. For control experiments, we treated cells identically with microwave-inactivated K-252a, microwave-inactivated staurosporine, or microwave-inactivated calphostin C (Wolniak and Larsen, 1992).

Microinjection Protocols

For microinjection experiments, stamen hairs were dissected from the filaments of six to 10 flowers (one to three influorescences) and mounted in 3% low-melt agarose in an open-topped chamber, as described in detail by Wolniak and Larsen (1992). The cells were impaled with microinjection pipettes that contained 10 μg/mL VRKRTLRRL at known times during prometaphase and metaphase. The tip of the injection pipette rarely penetrated more than 2 μm into the cell, and the impalement site was always located at the metaphase plate. Pneumatic pressure was used to inject VRKRTLRRL into the cytoplasmic compartment of the cell. One pneumatic pulse was used in these experiments; the pressure was 150 kPa, and the duration of the pulse was 500 msec. The estimated volume of the injection solution, based on calibration experments in low-viscosity oil (Wolniak and Larsen, 1992), was ~1 fL. Based on the ~12 pl cytoplasmic volume of the stamen hair cell (Wolniak and Larsen, 1992), the final concentration of VRKRTLRRL in the cytosol was 100 nM to 1 μM. Thus, the number of VRKRTLRRL molecules injected into the cytosol was 100,000 to 1,000,000. It is important to provide a sense of the magnitude of this injection. In stamen hair cells, we estimate conservatively (using both unpublished and published immunofluorescence data that we have generated; Larsen and Wolniak, 1993) that each half spindle comprises ~1000 microtubules and has a length of ~10 to 15 μm. Based on these numbers and accounting for nonuniformity of microtubule length in the spindle, there are ~10 to 15 million GTP binding sites on the tubulin molecules in the spindle alone. Thus, an injection of 10^5 to 10^6 VRKRTLRRL molecules into a stamen hair cell is, by comparison, a small number of substrate molecules.

During the course of developing a microinjection protocol that would preserve normal metaphase transit times with control solutions, it rapidly became apparent that excessive pressure of impalement could result in significant reductions in the transit times observed (Wolniak and Larsen, 1992). Therefore, the cell was always impaled with a "gentle" turn of the micromanipulator knob so that the tip of the injection pipette would cause only a minor deflection in the surface of the cell wall before it penetrated the plasma membrane. The extent of wall deflection was usually <2 μm, and then the tip of the injection pipette would suddenly penetrate the wall. Cells that were impaled with excessive force (that is, wall deflection >4 to 5 μm before pipette tip entry) were discounted from the sample. In addition to potential artifacts induced by excessive force of impalement, we found early on that subtle day-to-day differences in the injection protocol, or day-to-day differences in the cells themselves, could contribute to the metaphase transit times observed. To account for these kinds of problems, at least two microinjection experiments were performed each day, with cells injected at different time points during prometaphase or metaphase. At the beginning of this study, time points for injection after NEBD were selected on a random but predetermined basis. As the data set grew, on any given day cells were microinjected at a time point that would be a replicate of an earlier injection experiment, and a subsequent injection would be performed at a new time point. Usually, but especially in later trials, microinjections were performed to ensure that at least one metaphase transit time in a day's experiments was extended significantly, and the metaphase transit time of at least one other cell was the same as that of untreated or control cells. This strategy generated a number of replicate times of injection that were performed as much as 6 to 12 months apart. Control injections were performed in parallel, using the same strategy, with replicate experiments being performed on a number of different days.

For one set of microinjection controls, cells were impaled at identical times in prometaphase and metaphase and microinjected with carrier buffer only under identical conditions. Control microinjections were also performed using microwave-denatured VRKRTLRRL loaded into the cells with the same microinjection parameters at directly comparable times after NEBD. It is reasonable to suspect that even brief microwave irradiation disrupts the integrity of hydroxyl groups on amino acids. A third set of control microinjections was performed using the peptide VRKRALRRL. Again, the impalement injections were performed at identical time points during prometaphase and metaphase using the same injection parameters. The VRKRALRRL peptide, purified by FPLC, lacks the threonine phosphorylation site in the middle of the motif.

Microscopy and Video Image Recording

Progression through mitosis for all cells was recorded by time-lapse video, as described previously by Larsen and Wolniak (1990). Superimposed on the video image was a time–date generator signal to provide a timing record for each experiment. The timing of NEBD, anaphase onset, and cell plate vesicle aggregation was determined by replay of the videotape recordings. In addition to the video recordings, video images of all of the cells treated in these experiments were captured and signal-averaged with a Macintosh-based image processing system using the *Image* shareware package, developed at the National Institutes of Health (Bethesda, MD). For presentation of micrographs, computer-stored video images were photographed directly from one of the Macintosh monitors onto 6- × 7-cm Kodak T-Max 400 film, as described previously by Wolniak and Larsen (1992).

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