Mini-Review

Calcium Transients during Mitosis: Observations in Flux

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The idea that mitotic events are regulated by transients in the intracellular free calcium ion concentration ([Ca]i) is attractive (Hepler and Wolniak, 1983; Ratan and Shelanski, 1986; Poenie and Steinhardt, 1987; Wolniak, 1988). It is reasonable to imagine that several processes, such as formation/deformation of the mitotic apparatus, activation of a mecanochemical motor, transformation of spindle-associated membranes, and condensation and maturation of the chromosomes, are regulated in part by molecular processes that are stimulated by changes in the intracellular free [Ca]. Whether the presumed changes occur has been difficult to determine, due to the difficulty of measuring [Ca]i, as well as the difficulty of dissection of events that are important. In recent years, however, the introduction of Ca-specific probes (Tsien et al., 1982; Grynkiewicz et al., 1985) and improved detector systems have made possible the measurement of [Ca]i in single cells. Amidst the resulting flurry of reports, there are several that have specifically examined dividing cells, and although many indicate that changes in [Ca]i occur during mitosis, there is substantial disagreement concerning the timing, magnitude, and even direction of these transients. It is my purpose in this mini-review to focus on recent publications in which direct measurements of Ca transients in single dividing cells have been reported. Through a critical evaluation of the different observations, an attempt is made to sift out the meaningful trends and to derive a coherent story.

Ca Transients in Fertilized Eggs

Ca transients during the cell divisions that follow the fertilization of the egg, especially in marine invertebrates, have received considerable attention (Ridgway et al., 1977; Poenie et al., 1985; Yoshimoto et al., 1985; Silver and Inoue, 1987; Steinhardt and Alderton, 1988). The most tantalizing result comes from the study of Poenie et al. (1985) on the sea urchin Lytechinus variegatus that had been loaded with the penta(acetoxymethyl) ester of fura-2 (fura-2/AM).1 Beyond the large spike at fertilization, which serves as a test for the detection system, the authors observed five smaller elevations ranging from a mean basal level of 277 nM to peak heights between 410 and 665 nM. These correlated roughly with pronuclear migration, streak stage, nuclear envelope breakdown (NEB), onset of anaphase, and cytokinesis (Fig. 1). In an eagerness to embrace these results, it often goes unnoticed that the appearance of these transients, as stated by the authors, was unusual; normally the basal level of Ca approached 400 nM, and beyond the fertilization spike additional peaks were difficult to discern (Poenie et al., 1985) (Fig. 2). Also, the particular graph showing the sequential peaks was from a tetrapolar dividing egg, which is not normal. Subsequent work by Steinhardt and Alderton (1988) on the prophase/prometaphase transition provides more complete evidence supporting the occurrence of transients in Ca in association with NEB (Fig. 3). Here the resting level rises from 138 to 566 nM during a 1–3 min window preceding nuclear envelope dissolution. These observations are further strengthened by experimental studies showing that blockade of the natural Ca elevation inhibits NEB, whereas injection of Ca into inhibited cells can cause envelope dispersal to resume.

The occurrence of transients in Ca at different points in the mitotic cell cycle also finds support in the preliminary studies of Silver and Inoue (1987). Using the photoprotein aequorin that had been microinjected into sand dollar eggs, they reported increases in [Ca] that correlated with NEB, onset of anaphase and cytokinesis. Ridgway et al. (1977) also noted small increases in light emission from aequorin-injected medaka eggs roughly at the time of cleavage. However, quite different results have been obtained by Yoshimoto et al. (1985), in an examination of eggs of Hemicentrotus and medaka. From intracellular recording of single, aequorin-injected medaka eggs, they report a distinct downward deflection in light emission that correlates with furrowing, leading these authors to conclude that the [Ca] becomes reduced (<100 nM), not elevated, at cytokinesis (Fig. 4). No changes in light emission were observed in relation to the other phases of division. Finally, there is the earlier work of Rink et al. (1980), using Ca-sensitive microelectrodes, in which they failed to detect a change in intracellular [Ca] associated with any stage of mitosis or cleavage.

Uncertainties, therefore, still remain concerning the generality of Ca transients at the different stages of mitosis in eggs. The evidence thus far supports the occurrence of a Ca pulse preceding NEB (Steinhardt and Alderton, 1988). However, there is much less agreement for the other phases of division, especially the metaphase/anaphase transition and cleavage, which historically have attracted attention as likely points of Ca regulation. Despite the apparent utility of these eggs for studies of Ca transients during mitosis, there are substantial potential problems with the spatial location and magnitude of signals due to the large cytoplasmic volume relative to that of the mitotic apparatus. There is a possibility that Ca-dependent signals, either light emission from aequorin or fluorescence from fura-2, could arise from nonspindle

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1. Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; fura-2/AM, penta(acetoxymethyl) ester of fura-2; NEB, nuclear envelope breakdown.
Ca Transients in Cultured Mammalian Cells

The mammalian cells to be discussed here and the plant cells described above; they are cells in which the mitotic apparatus occupies a large fraction of the total cell volume and leaves relatively little nonspindle cytoplasm as a source of spurious signals. Of all the mitotic cells examined for Ca transients, those of the rat kangaroo (PtK1 and PtK2) have received the most attention (Keith et al., 1985a; Poenie et al., 1986; Ratan et al., 1986, 1988; Tsien and Poenie, 1986). Keith et al. (1985a) first reported for PtK2 cells loaded with the fluorescent indicator quin2/AM that the [Ca] declined from 53 to 28 nM during mitosis, reaching a low point at prometaphase, and remaining low until nuclear envelope reformation in telophase. In a subsequent study Ratan et al. (1986) used fura-2 that had been loaded by ATP permeabilization, and reported that the [Ca] became elevated from 46 to 90 nM during anaphase and remained high for several minutes. Video imaging revealed localized increases in the ion either at the spindle pole (up to 140 nM) or in a ring around the mitotic apparatus. Quite different results, however, were revealed by Poenie et al. (1986), who observed in PtK1 cells a sharp spike in [Ca], from 130 to 500-800 nM, that lasted only 20 s and was temporally correlated with the metaphase/anaphase transition (Fig. 5). Moreover, they showed by fluorescence imaging that the [Ca] increase occurred throughout the mitotic apparatus and was not confined to a particular region. These dramatic results have commanded considerable attention from those of us trying to decipher the calcium-mitosis connection, but their impact was lessened by the display in a subsequent review article by Tsien and Poenie (1986) of a PtK1 cell in which the Ca increase lasted 2 min, or about the length of anaphase, and was not therefore represented as a spike.

The situation becomes even more confusing when we consider the most recent publication by Ratan et al. (1988), in which they show spikes in the [Ca] (from 83 to 148 nM) that are not correlated temporally with the metaphase/anaphase transition (Fig. 6). Although 90% of the cells show a spike within 12 min of the metaphase/anaphase transition, they can occur before, during, or after the event. In addition, spikes were noted in interphase cells. Ratan et al. (1988) do show a slow increase in the baseline level of Ca (from a low of 53 to a high of 101 nM) starting in late metaphase and continuing through anaphase to cytokinesis.

Ca Transients in Fertilized Eggs of Lytechinus pictus

In Fig. 1, the calcium transients during the first cell cycle are shown and correspond to the following events: fertilization (f), pronuclear migration (pm), streak stage (s), nuclear envelope breakdown (nm), metaphase/anaphase transition (ma), and cleavage (c). Breaks in the trace represent times when the cell was observed to determine its phase of division. Fig. 2 shows a typical trace where the post-fertilization baseline is higher than in Fig. 1. The periodic transients beyond the first two are not evident, nevertheless the cell underwent normal cleavage. (Reproduced from Poenie et al., Nature [Lond.], 1985, 315:147-149, by copyright permission of MacMillan Journals, Ltd.)

Figures 1 and 2. Ca transients in fertilized eggs of the sea urchin Lytechinus pictus that have been loaded with fura-2 by microinjection. In Fig. 1, the calcium transients during the first cell cycle are shown and correspond to the following events: fertilization (f), pronuclear migration (pm), streak stage (s), nuclear envelope breakdown (nm), metaphase/anaphase transition (ma), and cleavage (c). Breaks in the trace represent times when the cell was observed to determine its phase of division. Fig. 2 shows a typical trace where the post-fertilization baseline is higher than in Fig. 1. The periodic transients beyond the first two are not evident, nevertheless the cell underwent normal cleavage. (Reproduced from Poenie et al., Nature [Lond.], 1985, 315:147-149, by copyright permission of MacMillan Journals, Ltd.)

Fig. 3. Prophase/prometaphase transition in eggs of Lytechinus pictus that have been loaded with fura-2 by microinjection. The eggs are subsequently activated by a 15-min treatment with ammonium chloride. A transitory increase in intracellular free Ca precedes the breakdown of the nuclear envelope (arrow). (Reproduced from Steinhardt and Alderton, Nature [Lond.], 1988, 332:364-366, by copyright permission of MacMillan Journals, Ltd.)
Without question, the results from PtK cells are confusing. Depending on the particular study, one finds that during mitosis: the [Ca] declines (Keith et al., 1985a), the [Ca] elevates (Ratan et al., 1986; Tsien and Poenie, 1986), the [Ca] spikes at the metaphase/anaphase transition (Poenie et al., 1986), the [Ca] spikes but not at the metaphase/anaphase transition (Ratan et al., 1988), and finally the [Ca] slowly increases from metaphase to anaphase (Ratan et al., 1988). The first result, namely that the [Ca] declines during mitosis (Keith et al., 1985a), can reasonably be eliminated from further consideration due to the likelihood that quin2, especially at the high concentration used (1 mM), caused severe buffering, and also that the dye as the AM ester had probably become sequestered in membranous compartments and was simply not reporting free intracellular Ca. We are still left with a variety of results that have all been obtained with fura-2, although using different loading procedures. It is disturbing that the results from different laboratories do not agree, but even more troubling is the disagreement in multiple studies from the same laboratory.

A further troubling aspect of the published results is the considerable disparity in the Ca values; Ratan et al. (1986, 1988) report values in which the peaks about equal the basal levels indicated by Poenie et al. (1986). Based on general information concerning Ca levels in other systems and on the affinity of the pumps that remove Ca from the cell, the values reported by Ratan et al. (1986, 1988) and also to a large extent those of Tombes and Borisy (1989) are unreasonably low. An increase of [Ca] to 150 nM is probably insufficient to act as a trigger to stimulate a process. If the primary target for Ca action is calmodulin, in which the high affinity binding sites exhibit a dissociation constant in vitro between 1 and 3 µM (Klee, 1980), then an increase of [Ca] to only 150 nM would not be expected to fill these sites and activate the protein. In a scheme involving "amplitude modulation," a [Ca] increase in the range of 500 to 800 nM (Poenie et al., 1986; Tsien and Poenie, 1986; Steinhardt and Alderton, 1988) seems realistic.

Two studies presented at the National Meeting of the American Society of Cell Biology in January 1989 may help explain some of the confusion that currently surrounds the observations from mammalian cells. Reports from Alderton et al. (1988), and Tombes and Borisy (1989), using Swiss 3T3 cells, indicate that brief spikes are observed only in the presence of serum in the culture medium; when the cells are cultured free of serum, they divide quite normally without spikes. Alderton et al. (1988) do note, however, that when the spikes occur they cluster close to the transition points of NEB and anaphase onset. In addition, because NEB in particular can be enhanced or retarded by experimentally modulating the intracellular [Ca]—either up, using nitr-5, a photolabile caged Ca compound, or down, using 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA)—Alderton et al. (1988) argue that Ca transients normally do occur even in the absence of serum but that they are below...
Ca Transients in Plants

One of the classic systems for the study of mitosis is the endosperm of the African blood lily, *Haemanthus*, and therefore it is not surprising that these cells have been used for Ca studies. Like the mammalian cells described above and the stamen hair cell considered below, endosperm cells may contain relatively little nonspindle cytoplasm when dividing, and therefore signals that are observed can be expected to come from the mitotic apparatus. Initial observations published by Keith et al. (1985b) on fluorescence images of quin2/AM-loaded cells indicated that the [Ca] increases from \( \sim 50 \text{nM} \) to a level two to threefold higher during anaphase, specifically in the chromosome-to-pole region. There are important reasons to be cautious about these results. Notably, the AM form of the dye was used, and as with the PtK2 cells studied by the same laboratory, we can anticipate problems with Ca buffering and dye sequestration that rendered the initial findings on PtK2 questionable. In support of the assertion that quin2 has become sequestered in membrane compartments, we note that the published images look quite similar to those obtained by Wolniak et al. (1980) on *Haemanthus* using chlortetracycline as a stain for membrane-associated Ca. It therefore seems questionable that free cytoplasmic Ca was being imaged with quin2 (Keith et al., 1985b).

The kinetics of Ca changes have been measured in dividing stamen hair cells of *Tradescantia* loaded by iontophoresis with the absorbance indicator dye arsenazo-III (Hepler and Callaham, 1987). Here the results indicate that the free [Ca] increases gradually in the mitotic apparatus during anaphase (Fig. 8). Temporal correlations show that the initial rise begins after the onset of anaphase, and continues gradually during the 10-15-min interval in which the chromosomes are moving towards the poles. The [Ca] plateaus when the chromosomes reach the poles and then declines to the resting level at the time of cell plate initiation. Unfortunately, the absolute levels of Ca cannot be stated because of uncertainties about dye concentration, dye-Ca stoichiometry, dye-protein binding, and non-dye absorbance. However, based on the absorbance changes that accompany a Ca injection that is just sufficient to stop cytoplasmic streaming (\( \sim 1 \mu M \)), we infer that the free concentration during late anaphase may approach 1-3 \( \mu M \), although often the observed increases are considerably smaller. As a Ca indicator in single cells, arsenazo-III lacks the sensitivity of the fluorescent dyes, and thus small but significant transients in the ion concentration may have occurred that were below the limit of detection. Nevertheless, it seems unlikely that global [Ca] spikes, of magnitude 0.5-10 \( \mu M \) or larger, occur at any time from midmetaphase through cytokinesis, thus providing evidence against their necessity as a mitotic regulator (Hepler and Callaham, 1987).

**Interpretation of the Results**

What do these different results mean? Are they due to biological differences between cells? Are they due to unreliable experimental methods? There is no compelling reason to assume that biological differences account for the variation in results. That the same laboratory can produce different results on the same cell type suggests that variations arise not from intrinsic biological difference in the tissues but from a combination of artifacts due to the culture and detection methods. We have already seen that the presence of FCS in the culture medium causes Ca spikes that appear to be unrelated to the events of mitosis. Regarding the detection of low levels of Ca, there are several problems that could confound the results. Because the indicator must interact with the free ion to produce a signal there will, of necessity, be some degree of buffering that may modify the events under observation. For example, the slowing of mitotic progression in *Tradescantia* by arsenazo-III is probably due to Ca buffering. This problem applies to all indicator-based studies of Ca, but may be more acute in the mitotic cell, in which the processes are relatively slow, occurring over extended periods of time, and where the changes in [Ca] may be small, especially when compared with those commonly encountered during such events as fertilization or muscle contraction. In addition, there is the matter of redundancy built into the mitotic system; the successful and correct separation of chromosomes is paramount to the existence of each and every
Figure 8. Metaphase through telophase in stamen hair cells of *Tradescantia* that have been loaded with the absorbance indicator dye arsenazo III. A gradual increase in the [Ca] is observed to begin immediately after the metaphase/anaphase transition (M/A), and continues while the chromosomes move to the poles. Thereafter, the Ca gradually declines, reaching basal levels at about the time of cell plate formation (CP). The top two traces show two separate dye-injected cells while the lower two traces are from dividing cells lacking dye. (Reproduced from Hepler and Callaham, *J. Cell Biol.*, 1987, 105:2137–2143.)

cell. When one operating system becomes stressed, others may emerge to assist and insure success. Thus it may be that a Ca pulse under one set of conditions may act as a sufficient stimulant, whereas under other conditions it could be substituted by some other process.

Notwithstanding these general matters, which make the measurement of Ca particularly difficult in the mitotic cell, there is concern that the existing measuring methods themselves contain problems that have not been sufficiently well considered. While fully acknowledging the efficacy of the fluorescent dyes and the enormous positive impact that they have had on the Ca field, it must be recognized that they are not without shortcomings. First, the dyes may be sequestered into membranous compartments and not indicate cytoplasmic free Ca (Malgaroli et al., 1987). The use of dyes in their AM form, although convenient, probably exacerbates compartmentation. We have already noted this problem in discussing the results obtained with quin2 on both PtK and *Hae-manthus* endosperm cells. But fura-2 and indo-1 also compartmentalize, even as the free anion form of the dye; in plants these dyes can quickly become sequestered into the vacuole, effectively reducing the dye concentration in the cytoplasm below usable levels (Callaham, D. A., and P. Hepler, unpublished observations). Further, mention should be made that in PtK cells, suboptimal culture temperatures must be used to prevent dye compartmentation and extrusion from the cell (Poenie et al., 1986; Ratan et al., 1988). The problem of dye sequestration/excretion is becoming more widely appreciated, but it is important to realize that it is probably worse in studies of mitosis where culture times are prolonged while one is waiting for a particular event such as the onset of anaphase.

Additional problems with the indicator dyes result from photobleaching (Becker and Fay, 1987), the presence of uncleaved ester (Scanlon et al., 1987), viscosity (Konishi et al., 1988; Tsien and Poenie, 1986), protein binding (Konishi et al., 1988), and light/dye toxicity (Callaham, D. A., and P. Hepler, unpublished observations). Some of these difficulties such as protein binding are not restricted to the fluorescence indicators since studies with arsenazo-III indicate that as much as 80–90% of the dye in muscle cells may be complexed with protein (Beeler et al., 1980). It is not appropriate in this review to detail these problems; suffice it to say for the fluorescence indicators that the first four will reduce the sensitivity of the signal or lower the apparent [Ca], while the last may inhibit mitosis and damage the cell. These are not trivial concerns because, as pointed out by Konishi et al. (1988) in studies using fura-2, the cumulative errors resulting from viscosity and protein binding can effectively reduce the apparent free [Ca] by a factor of 10. Again, in studies of mitosis, the necessity of culturing cells for appreciable time periods may increase protein binding and thus further decrease the dye-Ca response. These considerations may help explain some of the very low values in [Ca] that have been reported. The final problem of light/dye toxicity has recently become apparent to us in studies of indo-1-loaded stamen hair cells of *Tradescantia* (Callaham, D. A., and P. Hepler, unpublished observations) in which we find that the combination of dye (50–100 μM) and light (350 nm [10 nm band width] at 70 μW/mm²), even at quite moderate levels, will arrest cells in mitosis.

Although there are variations in the data, some of which may arise from problems in the existing measuring methods, there are, nevertheless, trends that permit some tentative conclusions about the presence and meaning of Ca transients during mitosis. Despite the attractiveness of "trigger pulses" of Ca as a general regulatory mechanism for key events during mitosis, the data obtained from direct measurements, especially for the metaphase/anaphase transition, do not provide convincing support for this hypothesis. In mammalian cells the spikes do not appear to correlate temporally with the metaphase/anaphase transition (Ratan et al., 1988; Tombs and Borisy, 1989), and more important, they now seem to be caused by the presence of serum in the culture medium (Alderton et al., 1988; Tombs and Borisy, 1989). Moreover, experimentally raising and lowering the [Ca], while profoundly modulating NEB, by contrast has much less effect on the metaphase/anaphase transition (Alderton et al., 1988). Finally, spikes in the [Ca] are not observed during mitosis in plant cells (Hepler and Callaham, 1987). Transient elevations, however, are observed to precede NEB in the sea urchin, *Lytechinus*, and would appear to have a regulatory role (Steinhardt and Alderton, 1988). This important point needs to be examined in other cell types. The Ca signal at cytokinesis remains unclear with both increases (Poenie et al., 1985; Ridgway et al., 1977; Silver and Inoue, 1987) and decreases (Yoshimoto et al., 1985) having been reported at cleavage furrow formation.

An emerging trend that deserves attention is the presence of gradual increases in [Ca] during metaphase and anaphase (Hepler and Callaham, 1987; Ratan et al., 1988; Tombs and Borisy, 1989). These have been observed in three different cell types (PtK2, Swiss 3T3, *Tradescantia* stamen hair), using two different detection probes (fura-2, arsenazo-III), although, interestingly they have not been observed by Alderton et al. (1988) in their examination of the fura-2-loaded Swiss 3T3 cells. In those examples, where the increase has been reported there is still some disagreement as to when the rise begins, with the studies of Ratan et al. (1988) and Tombs and Borisy (1989) on PtK and Swiss 3T3 cells, respectively, indicating a rise beginning during metaphase and extending into anaphase, whereas in *Tradescantia* the rise appears to begin immediately after the onset of anaphase (Hepler and Callaham, 1987). The close correlation, especially in *Trad-*
escantia, between the Ca rise and anaphase A provides support for the idea that the ion contributes to the regulation of chromosome motion, perhaps through controlled depolymerization of spindle MTs (Zhang et al., 1988). Despite a semblance of agreement on the occurrence of gradual increases in [Ca] during metaphase and anaphase, it would still be prudent to receive these results with some caution, especially given their variability. Further experimentation is clearly needed to confirm or reject these findings.

Even if we accept the occurrence of gradual increases in [Ca] that begin in metaphase, it is not clear that they could act as a switch to cause anaphase onset, an event that is abrupt and simultaneous throughout the mitotic apparatus and would seem by its very nature to require a correspondingly abrupt signal. In view of these considerations, we must carefully rethink our ideas about the control of the metaphase/anaphase transition. It is, of course, possible that regulatory pulses do occur, but because they are highly localized, for example, at the kinetochore where microtubule polymerization/depolymerization is presumed to occur, they might be extremely difficult to detect (Alderton et al., 1988).

Alternatively, Ca pulses may not be the direct regulator of the transitional events of mitosis. Instead gradual increases in [Ca] may create localized gradients within the cell, and these could activate processes that are themselves spatially defined by the position and magnitude of the gradient. Invaginating elements of endoplasmic reticulum in the mitotic apparatus of many different cell types (Hepler and Wolniak, 1984) provide a system that could release Ca in specific regions, for example at the kinetochore, and thus establish these hypothetical local gradients. Although it is usually assumed, based on studies in which mitotic events are blocked by the introduction of a Ca-buffer, that the buffer damps the Ca pulse (Izant, 1983; Alderton et al., 1988; Steinhardt and Alderton, 1988), an alternative explanation is that the buffer dissipates a local gradient (Spoksnijder et al., 1989). Recent studies on fucoid eggs, for example, reveal that substituted BAPTA buffers with a dissociation constant that is defined by the position and magnitude of the gradient. In view of these considerations, we must carefully rethink our ideas about the control of the metaphase/anaphase transition. It is, of course, possible that regulatory pulses do occur, but because they are highly localized, for example, at the kinetochore where microtubule polymerization/depolymerization is presumed to occur, they might be extremely difficult to detect (Alderton et al., 1988).

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Conclusion
The direct examination of Ca transients during cell division is in its infancy; we are still wrestling with a variety of problems that confound the measurement of small amounts of Ca in single dividing cells. Nevertheless, it is crucial to establish whether Ca transients occur during mitosis, as their existence could relate in a fundamental way to the control of cell division. Although uncertainty surrounds many of the published results, there are some exciting new findings and these deserve further attention. In one sense the field may have been slowed by preconceived ideas, for example that the pulses of Ca would occur at key points and stimulate subsequent events. When a few initial studies reported these pulses, the mitotic control issue seemed destined for an early solution. However, subsequent studies provide alternative results that contradict the early reports. The solution is thus not as simple as first conceived, and the difficult but exciting task of establishing the true presence and significance of Ca transients during mitosis largely lies ahead.

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