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Intracellular free calcium oscillations in normal and cleavage-blocked embryos and artificially activated eggs of *Xenopus laevis*

Thomas J. Keating*, R. John Cork and Kenneth R. Robinson

Department of Biological Sciences, 1392 Lilly Hall, Purdue University, West Lafayette, IN 47907-1392, USA

*Author for correspondence

SUMMARY

We have measured levels of intracellular free calcium ($[Ca^{2+}]_i$) in albino *Xenopus laevis* embryos using recombinant aequorin and a photon-counting system. We observed sinusoidal oscillations in $[Ca^{2+}]_i$ that had the same frequency as cleavage, with cleavage occurring when $[Ca^{2+}]_i$ was lowest. An increase in calcium was seen to precede first cleavage. The cyclic changes in calcium were superimposed on a secondary pattern that increased, peaked between third and fifth cleavages and then slowly declined to a level similar to that measured before first cleavage. The amplitude of the oscillations was small during the first few cleavages but became larger with each cycle, with the largest oscillations occurring when the secondary pattern peaked (between third and fifth cleavage). As the secondary pattern declined, the amplitude of the oscillations also became smaller. The oscillations are due to release of calcium from intracellular stores, since the signal was the same in calcium-free solution as in normal medium.

When cleavage was blocked with the microtubule-disrupting drugs colchicine or nocodazole, the $[Ca^{2+}]_i$ oscillations persisted. Calcium oscillations of a similar magnitude and frequency were also present in artificially activated eggs. The secondary pattern was different in cleavage-blocked embryos and artificially activated eggs, the baseline increasing until about the third cycle and then remaining elevated for the rest of the recording (>8 hours).

By fixing embryos at various points in the calcium cycle, we determined that mitosis began shortly after calcium levels reached their peak and was complete before the calcium level dropped to its lowest point. The fact that the calcium oscillations persist when nuclear division is not occurring suggests that either they operate independently of any downstream events that they might control or they are related to other cyclic activities in the eggs and embryos such as cycling of pH_i, MPF, or surface contraction waves.

When aequorin was injected into individual blastomeres of 64-cell embryos, the shape of the signal was substantially different from that seen in recordings from whole embryos. Compared to the whole-embryo recordings, the signal from a subregion of the embryo rose more quickly and had a slower, biphasic decline. These differences indicate that $[Ca^{2+}]_i$ increases are not occurring uniformly across the embryo but are spatially localized, perhaps progressing as waves.

Key words: calcium oscillations, cell division, *Xenopus laevis*, recombinant aequorin

INTRODUCTION

It has been suggested that changes in intracellular free calcium ($[Ca^{2+}]_i$) may act to initiate and regulate the events of cell division. Despite numerous studies aimed at showing a role for $[Ca^{2+}]_i$ in mitosis and cytokinesis, the evidence is still inconclusive (see reviews by Hepler, 1989; Petzelt and Hafner, 1989).

The eggs and embryos of the frog *Xenopus laevis* have been used extensively as a model system for studying the cell cycle. Although there is indirect evidence supporting the idea that increases in $[Ca^{2+}]_i$ are necessary for cell cycle progression in *Xenopus* embryos, direct measurements of calcium levels have so far produced mixed results.

Baker and Warner (1972) buffered $[Ca^{2+}]_i$ in *Xenopus* embryos by injecting solutions of calcium and EGTA. They concluded that changes in calcium were not required for cleavage because the embryos continued to divide when $[Ca^{2+}]_i$ was buffered at 100 nM or above. Rink et al. (1980) also reported that embryos continued to cleave if $[Ca^{2+}]_i$ was held at 100 nM with the chelator BAPTA, again suggesting that calcium increases are not necessary for division. However, since calcium levels were not measured in chelator-injected embryos, it is not known if any normal calcium increases were actually blocked under these conditions.

Han et al. (1992) observed that *Xenopus* blastomeres stopped cleaving when injected with heparin or antibodies to the phospholipid PIP_2, both of which would be expected to interfere with the IP_3 pathway and hence inhibit release of calcium from intracellular stores. Both Snow and Nuccitelli (1993) and Miller et al. (1993) injected *Xenopus* embryos with a range of BAPTA derivatives having different affinities for

Ca^{2+} and found that the relatively weak buffer, dibromo-BAPTA (K_D for Ca^{2+} ~ 1.5 μM), was the most effective at inhibiting cleavage. They interpreted this result to mean that at one or more points in the cell cycle there are localized gradients of calcium that are necessary for cell cycle progression and that the peak concentration of these gradients is several micromolar. A similar approach was taken by Sullivan et al. (1993) to demonstrate that fusion of nuclear membrane vesicles in *Xenopus* egg extracts requires a gradient of calcium. In all of the above cases, however, the role of calcium was determined indirectly, being inferred from the results of buffer additions.

Several groups have measured $[Ca^{2+}]_i$ in *Xenopus* embryos using calcium-specific microelectrodes. In addition to the buffer-injection experiments mentioned above, Rink et al. (1980) measured $[Ca^{2+}]_i$ during two rounds of cleavage in *Xenopus* embryos but failed to see any $[Ca^{2+}]_i$ changes during this period. Busa and Nuccitelli (1985), in a study of the calcium wave at fertilization using calcium microelectrodes, showed one recording of $[Ca^{2+}]_i$ that lasted through first cleavage. No change in $[Ca^{2+}]_i$ was seen at the time of cleavage.

In a more extensive study of calcium levels during early development in *Xenopus*, also using calcium microelectrodes, Grandin and Charbonneau (1991) reported seeing oscillations in $[Ca^{2+}]_i$ that had the same frequency as cleavage. As they did not see any oscillations in embryos that were prevented from cleaving by the addition of nocodazole or in artificially activated eggs, Grandin and Charbonneau (1991) concluded that the calcium oscillations that they measured were related to some specific event of mitosis or cytokinesis. If calcium oscillations occur only when the embryo is cleaving then it should be possible to narrow down the number of events of cell division that might be controlled by these $[Ca^{2+}]_i$ oscillations by eliminating from consideration the events that occur in nondividing embryos and eggs. Given the potential importance of the findings of Grandin and Charbonneau (1991) and the fact that their results contradicted earlier studies (Rink et al., 1980; Busa and Nuccitelli, 1985), we thought that a further investigation was warranted.

In an attempt to clarify this situation we chose to measure $[Ca^{2+}]_i$ in *Xenopus* embryos using the calcium-sensitive photoprotein aequorin and a photon-counting system. To avoid the problem of the eggs' pigment absorbing varying amounts of light during the cleavage cycle, we used eggs from albino *Xenopus* for these studies. We have confirmed the presence of $[Ca^{2+}]_i$ oscillations in cleaving embryos but, in contrast to Grandin and Charbonneau (1991), we have found similar oscillations in artificially activated eggs as well as in embryos in which cleavage was blocked with the microtubule-disrupting drugs nocodazole and colchicine.

A preliminary report of this work was presented at the 1992 meeting of the American Society for Cell Biology (Keating and Robinson, 1992). During the preparation of this manuscript the results of another study of $[Ca^{2+}]_i$ in *Xenopus* embryos using aequorin were published (Kubota et al., 1993). The results published here confirm and extend those of Kubota et al. (1993), showing that calcium oscillations occur in artificially activated eggs as well as in embryos. Further, we demonstrate that the increases are due to release of calcium from intracellular stores, since the oscillations persist in calcium-free solutions. Finally, we provide evidence to suggest that the calcium oscillations do not occur uniformly over the multicellular embryo but are localized and might travel as waves.

MATERIALS AND METHODS

Albino *Xenopus laevis* frogs (Nasco, Ft. Atkinson, WI) were induced to ovulate by injection of 500 i.u. of human chorionic gonadotropin (Sigma Chemicals, St Louis, MO). Ten to fourteen hours after injection, eggs were stripped from the females into dry 60 mm ×15 mm Petri dishes. For fertilization, the eggs were rubbed with macerated testis and the dish was subsequently filled with 0.1× modified Ringer's solution (0.1× MR; concentrations (in mM): 10 NaCl, 0.2 KCl, 0.1 $MgSO_4$, 0.2 $CaCl_2$, 0.5 NaHEPES, 0.01 EDTA, pH 7.5; Newport and Kirschner, 1982).

Fifteen minutes after fertilization, a dozen or so newly fertilized embryos were manually dejellied, removing the outer, sticky layers of jelly but leaving the inner, firm layer intact. Before microinjection, activation of the eggs was confirmed by noting the slight flattening of the animal pole, which is a sign of the cortical contraction that follows activation. Fertilized embryos were then injected with 5-10 nl of a 1 mg/ml solution of recombinant *h*- or *f*-aequorin (Shimomura, 1991), provided by Dr O. Shimomura (Marine Biological Laboratory, Woods Hole, MA) and his collaborators Dr Y. Kishi (Harvard University) and Dr S. Inouye (Chisson Corp., Yokohama, Japan). Recombinant *h*- and *f*-aequorin have nearly identical calcium sensitivities (Shimomura, 1991) and therefore were used interchangeably.

Microinjection pipettes were pulled from glass tubing with a capillary fiber inside (1B150F-6, World Precision Instruments, Sarasota, FL); the tip of each pipette was then beveled at a 45° angle until the inside diameter of the tip was 5 μm. Embryos were placed in small wells in an agarose-coated Petri dish to hold them in place during injection. Solution was injected by air pressure generated by a glass syringe, the volume being estimated by measuring droplets suspended from the end of the pipette before and after injection. Embryos were not used for recording if large amounts of yolk leaked from the site of the injection. The recordings from dividing embryos are included in the results here only if the embryos developed into normal tadpoles.

Photon counting was performed as previously described (Cork et al., 1987), the design of the chamber being such that the animal hemisphere faces the photocathode. Photon-generated pulses were counted with a PC-LPM-16 data acquisition card (National Instruments, Austin, TX) running in an IBM-PC/XT. The total number of photons collected each second was stored during the experiments, generating up to 40,000 data points in some cases. To import the data from an experiment into our graphing program (Cricket Graph, Computer Associates, San Diego, CA), we averaged together 10-30 consecutive points and plotted the condensed data.

The signal without an embryo in the chamber was <1 photon/second and most of our recordings had signals of >100 photons/second. In most experiments the recording chamber was perfused with 0.1× MR at a rate of approximately 0.05 ml/minute, while the chamber itself has a volume <0.5 ml. Experiments were conducted at 20-22°C.

To disrupt microtubules and thereby prevent cleavage, fertilized eggs were injected with 10-15 nl of a 40 mM solution of colchicine (Sigma) in distilled water. Assuming that the non-organellar cytoplasmic volume of a *Xenopus* egg is 400-500 nl, the final concentration of colchicine was approximately 1 mM. For nocodazole treatment, fertilized embryos were perfused in the recording chamber with 0.1× MR plus 1-10 μg/ml nocodazole (Aldrich Chemical, Milwaukee, WI); nocodazole stock solution was 3 mg/ml in DMSO.

Eggs that were to be artificially activated were submerged in 0.1× MR without addition of macerated testis. Such eggs were activated when they were injected with aequorin solution.

The decrease in light output that is seen during later cleavages in fertilized embryos (Fig. 1) is due mainly to a decrease in $[Ca^{2+}]_i$ rather than consumption of the injected aequorin. Although aequorin molecules are irreversibly consumed when they bind calcium and emit light, the rate of consumption at resting levels is quite low. Our in vitro calibrations (performed as described by Cork et al., 1987) of *h*-aequorin indicate that only 10^{-6} of the photoprotein that is present will be consumed per second when $[Ca^{2+}]_i$ is 300 nM. At that rate, only 2-3% of the total aequorin would be consumed in a 6-9 hours recording, but because $[Ca^{2+}]_i$ goes above resting levels, the consumption of aequorin during an actual experiment will be slightly higher. In one embryo, the fraction of aequorin that was consumed during the first 7 hours following injection was measured and was found to be 4.2% of the total amount present. This particular measurement spanned the time from 1 to 8 hours post-fertilization and was similar to the recordings presented in this report.

Embryos that were to be bathed in calcium-free solution were dejellied completely, either with forceps or by treatment with 2% cysteine (pH 7.8). The calcium-free medium consisted of 0.1× MR solution plus 5 mM EGTA (pH 7.5), making the free calcium concentration of this solution <1 nM. In some experiments the embryo was initially bathed in normal MR and the solution was changed to calcium-free MR during the recording, while in other cases the embryo was bathed in calcium-free MR from the beginning of the recording. The aequorin recordings were equivalent in either case.

For experiments in which the nuclei were examined, the embryos were dejellied in 2% cysteine (pH 7.8) 15 minutes after fertilization. The embryos were then injected with 5-10 nl of aequorin solution and placed in the recording chamber. The recordings were stopped at various points in the calcium oscillation cycle and the embryo was removed from the chamber. The embryo was placed on a glass slide and squashed with a 22 mm × 22 mm coverslip in the presence of 4 μl of fixative containing the DNA-labeling dye, Hoechst 33342 (Murray, 1991).

RESULTS

Calcium levels in fertilized albino eggs

We have monitored intracellular free calcium ($[Ca^{2+}]_i$) in fertilized eggs from albino *Xenopus* and have observed oscillations in $[Ca^{2+}]_i$ with a period of approximately 30 minutes, the same as the rate of cleavage (Fig. 1). These oscillations were seen in all 12 long-term recordings from embryos that developed into normal tadpoles.

The average peak-to-peak amplitudes of the oscillations preceding each cleavage is shown in Fig. 2 (filled bars). The amplitudes are normalized against the resting level of each recording to correct for differences in the amount and activity of the aequorin that was injected. The resting level was taken to be the lowest level of light output obtained during the recording.

The oscillations were superimposed on a secondary pattern that rose steadily, peaked between the third and fifth cleavages, and then declined during the subsequent cleavages until it reached a level similar to that measured before first cleavage (Fig. 1). This secondary pattern was consistent, being discernible in all of the long-term (6-10 hours) recordings from healthy embryos.

The calcium oscillations that we measured were not brief, transient increases in $[Ca^{2+}]_i$ but rather were slowly varying changes that were almost sinusoidal in appearance. We occasionally saw brief (10-20 second) increases in light output that were superimposed on the steady changes, but these were infrequent and did not always occur at the same points in the cell cycle. However, the fact that we saw these changes indicates that our system would be able to detect fast calcium transients if they were occurring consistently.

The amplitude of the calcium increase at each cycle was related to the secondary pattern of Ca^{2+} changes. The largest oscillations were seen between the second and sixth cleavage, when the underlying calcium levels were highest (Figs 1 and 2). As the secondary calcium level increased in early cleavages or decreased in later cleavages, the amplitude of the oscillations became proportionately larger or smaller (Figs 1 and 2).

Although the embryo from which the aequorin signal was being measured could not be continuously monitored, we were able to determine the approximate times of cleavage by observing sibling embryos or by occasionally stopping the recording and directly examining the embryo in the chamber. From these observations we determined that cleavage occurs 15-20 minutes after the previous peak of $[Ca^{2+}]_i$ (Fig. 1A). Also, the furrows at first and second cleavage take 5-10 minutes to travel from the animal pole to the equator.

To determine the timing of nuclear events of the cell cycle relative to the calcium changes, we performed a series of experiments in which the embryo was removed from the recording chamber at a particular point in the Ca^{2+} oscillatory cycle and squashed on a slide in the presence of fixative and DNA-labeling dye. By examining these slides we were able to determine that mitosis begins several minutes after the calcium levels reach their peak and is complete within ten minutes, before $[Ca^{2+}]_i$ drops to its lowest level (Fig. 1A). S-phase is coincident with the rising phase of the calcium oscillations.

We performed calibrations on four embryos according to the method described by Cork et al. (1987). We took the resting level to be the lowest level of light output obtained during the recording and from the calibration we determined that the resting $[Ca^{2+}]_i$ level is 300-350 nM, which is in good agreement with previous values obtained with calcium microelectrodes (Rink et al., 1980; Busa and Nuccitelli, 1985; Grandin and Charbonneau, 1991). We can estimate the magnitudes of the calcium changes by assuming that they are occurring uniformly over the surface of the embryos. The largest Ca^{2+} oscillations, which usually were between the second and sixth cleavage, had an amplitude of approximately 50 nM. The right-hand scale on each trace indicates approximate $[Ca^{2+}]_i$ levels, based on the assumptions that the resting level is 300 nM and that light output increases $10^{2.5}$-fold for a tenfold increase in $[Ca^{2+}]_i$ (Blinks et al., 1982). Since the calcium changes seem to be localized (see below), these values represent the lower limits of the increases.

Calcium oscillations persist in calcium-free medium

The calcium increases could be due to influx from the extracellular medium. This possibility was eliminated by the finding that the Ca^{2+} oscillations continued with similar amplitudes when the embryos were bathed in calcium-free medium (Fig. 3). The amplitudes of the Ca^{2+} oscillations in this embryo are somewhat smaller than the examples shown in Fig. 1 but are still within the range of amplitudes seen in other embryos in calcium-containing solution. The amplitudes of the Ca^{2+} oscillations in this embryo were similar whether it was bathed in regular or calcium-free solution (Fig. 3), indicating that the

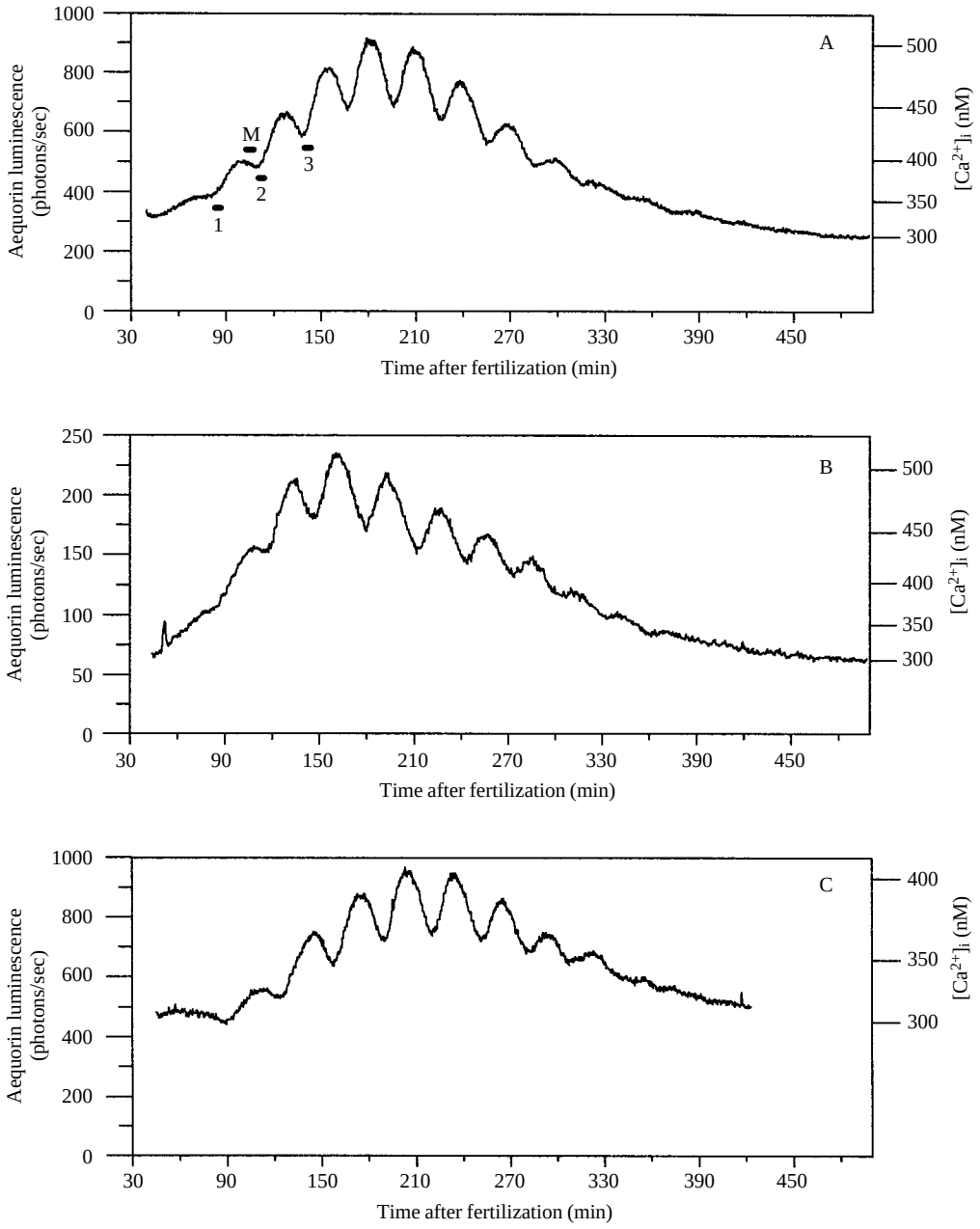

Fig. 1. (A-C) Aequorin luminescence in untreated, fertilized eggs from albino *Xenopus*. Three examples of recordings from fertilized eggs, from before first cleavage until after twelfth cleavage. In A the bars labeled 1, 2 and 3 indicate the approximate times during which the first three cleavages occurred in the animal hemisphere. Also in A, the bar labeled M indicates when mitosis occurs relative to the calcium oscillations. Approximate $[Ca^{2+}]_i$ levels are shown on the right-hand side of each graph.

smaller $[Ca^{2+}]_i$ increases were not related to the absence of calcium in the external solution but were due to normal variability. The cumulative results of recordings from 15 embryos bathed in calcium-free solution are shown in Fig. 2 (hatched bars). These results show that there are no consistent differences between the $[Ca^{2+}]_i$ signals obtained from embryos bathed in regular or calcium-free solution.

Calcium oscillations in nondividing embryos and activated eggs

In order to investigate the relationship between the Ca^{2+} oscillations and mitosis, we injected the microtubule-disrupting drug, colchicine, into fertilized, aequorin-injected eggs and measured their luminescence (Fig. 4). Contrary to the results of Grandin and Charbonneau (1991), who did not see calcium changes in cleavage-blocked embryos, the nondividing embryos showed oscillations in $[Ca^{2+}]_i$ that were similar to those seen in cleaving embryos (Fig. 4). Comparable results were observed in four out of five colchicine-injected embryos as well as all four embryos that were bathed in the microtubule-disrupting drug, nocodazole, during recording. Fig. 6 (below) shows the average amplitudes of the oscillations of light output in the eight cleavage-blocked embryos (filled bars).

We also monitored $[Ca^{2+}]_i$ in artificially activated eggs, since they have been shown to have periodic surface contraction waves (Hara et al., 1980) as well as oscillations of pH_i (Webb and Nuccitelli, 1981) and MPF activity (Gerhart et al., 1984). In six out of seven prick-activated eggs we observed oscillations of $[Ca^{2+}]_i$ that were similar to those seen in fertilized embryos (Fig. 5). The aequorin signals from artificially

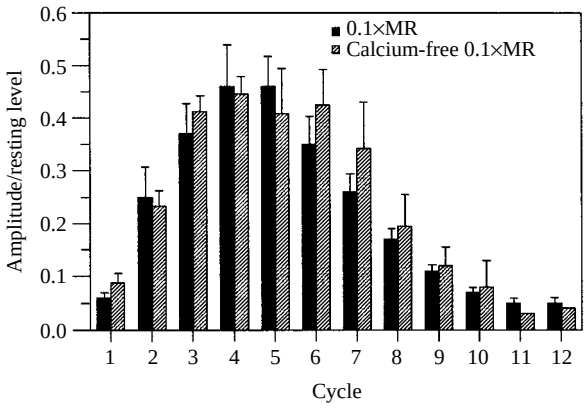

Fig. 2. Average peak-to-peak amplitudes of the Ca^{2+} oscillations associated with each cleavage cycle, from first to twelfth cleavage, for embryos bathed in regular 0.1× MR (filled bars) or Ca^{2+}-free 0.1× MR (hatched bars). The amplitudes of the oscillations have been divided by the resting level of light output to correct for differences in the amount and activity of aequorin injected into each embryo. Cycle 1 is the [Ca^{2+}]$_i$ oscillation preceding first cleavage, cycle 2 precedes second cleavage, and so on. Error bars indicate s.e.m.; a point without an error bar consists of a single measurement.

activated eggs differed from those of fertilized embryos in that there were more brief spikes superimposed on the oscillations. The average amplitudes of the increases in light output at each cycle in the six artificially activated eggs are shown in Fig. 6 (hatched bars).

A consistent observation in the recordings from embryos treated with microtubule-disrupting drugs and from prick-activated eggs was that the oscillations were superimposed on a secondary pattern of Ca^{2+} increase during the first three cycles and remained elevated for the duration of the recording (Figs 4 and 5).

The relationship between the amplitude of the oscillations and the secondary pattern of calcium increase was maintained in nondividing embryos and eggs. The calcium oscillations became larger as the underlying [Ca^{2+}]$_i$ level rose in the first two or three cycles. After the third cycle the secondary pattern remained elevated and the amplitude of the changes was fairly constant (Figs 4, 5 and 6).

[Ca^{2+}]$_i$ signal from a subregion of the embryo

We wanted to know whether the calcium changes occur uniformly throughout the embryo or if they are spatially localized, perhaps progressing as waves. To investigate this, we recorded the light output from albino embryos in which a single blastomere had been injected with aequorin at the 64-cell stage (Fig. 7). As aequorin is a 20 kDa protein, it does not pass through gap junctions, although the injected blastomeres continued to divide. The shape of the signal from a small subregion of the embryo (the progeny of the injected blastomere) was substantially different from the whole-embryo recordings at the same stage. In recordings from embryos injected in single blastomeres the calcium oscillations rose quickly and had a slow, biphasic decline (Fig. 7). The biphasic calcium decreases were discernable in six out of seven experiments in which oscillations were seen. In four other cases the amount of light emitted from the blastomere was too small to allow the oscillations to be seen.

DISCUSSION

Measurement of [Ca^{2+}]$_i$ in *Xenopus* embryos and eggs using aequorin or calcium microelectrodes

A major advantage of using aequorin rather than calcium microelectrodes to monitor calcium in cells is that aequorin has a nonlinear response, its luminescence increasing more than a 100-fold for a tenfold increase in [Ca^{2+}]$_i$ (Blinks et al., 1982). This exaggerated response improves the chances of measuring small or localized changes in [Ca^{2+}]$_i$ that might be missed by other methods.

An earlier attempt to measure [Ca^{2+}]$_i$ in *Xenopus* embryos with aequorin (Baker and Warner, 1972) was hindered by the use of eggs from wild-type frogs. Wild-type eggs have pigment in their cortex but the new membrane that is added in the furrow during cleavage does not have pigment associated with it. Baker and Warner (1972) reported seeing an increase in luminescence during cleavage but suggested that it may have been due to a decrease in light absorption in the cleavage furrow. To avoid this problem we used eggs from albino *Xenopus* females, which, although not transparent, are free of cortical pigments.

The results of this study and that of Kubota et al. (1993)

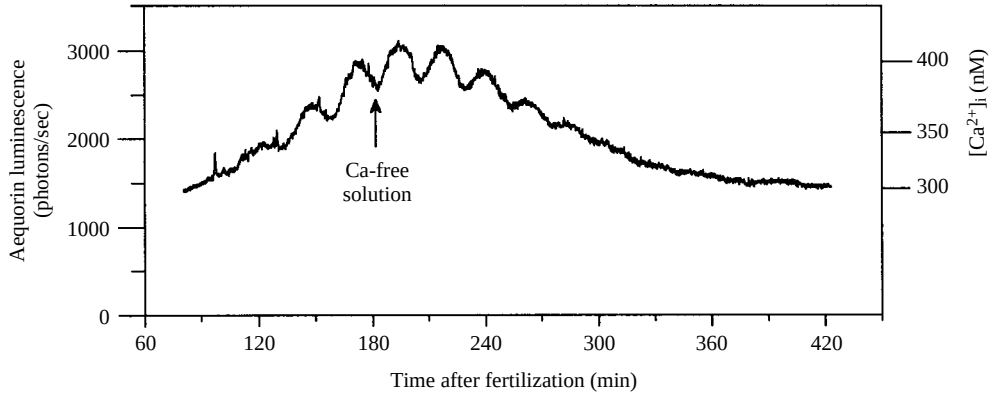

Fig. 3. Aequorin recording from a fertilized embryo that was bathed in calcium-free medium beginning at 180 minutes after fertilization (arrow).

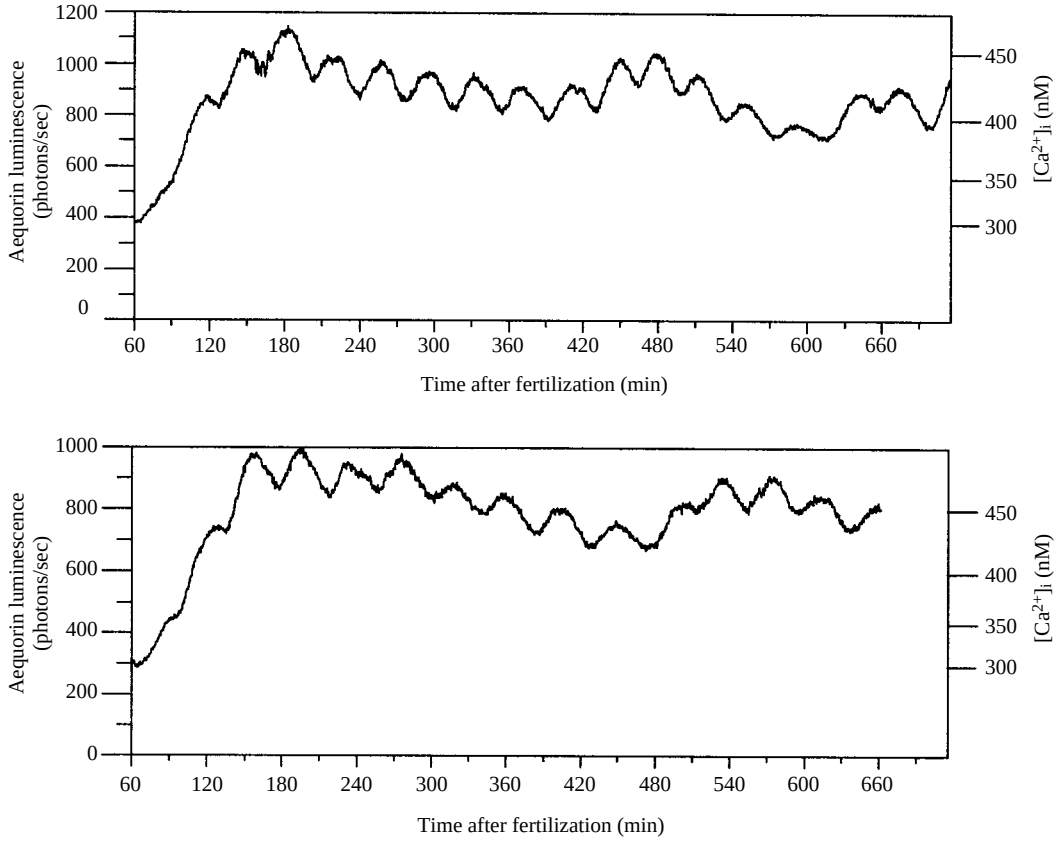

Fig. 4. Two examples of aequorin luminescence in cleavage-blocked embryos. Fertilized embryos were injected with aequorin and the microtubule-disrupting drug colchicine (final concn of colchicine 1 mM) before first cleavage. Although the colchicine-injected embryos did not divide, they had calcium oscillations that were similar to those seen in normal, untreated embryos.

show that $[Ca^{2+}]_i$ oscillates in cleaving embryos at the same rate as cleavage. In contrast to Grandin and Charbonneau (1991), our measurements and those of Kubota et al. (1993) reveal that there are similar calcium changes occurring in cleavage-blocked embryos as well. In addition, we found that prick-activated eggs also have oscillating Ca^{2+} levels.

The discrepancies between the results of previous measurements of $[Ca^{2+}]_i$ (Rink et al., 1980; Busa and Nuccitelli, 1985; Grandin and Charbonneau, 1991) and more recent results (this study; Kubota et al., 1993) might be due to the use of aequorin instead of calcium-sensitive microelectrodes. The calcium changes that we and Kubota et al. (1993) have measured have relatively small amplitudes, which means that the Ca^{2+} increases either are modest increases that are occurring globally or are larger but are localized. In both situations, it is more likely that the changes will be seen with aequorin than with calcium microelectrodes.

Indirect evidence favoring a role for $[Ca^{2+}]_i$ in the cell cycle of *Xenopus* embryos

There is some evidence, from injections of derivatives of the calcium chelator BAPTA, that gradients of calcium are required for progress of the cell cycle in *Xenopus* embryos (Han et al., 1992; Snow and Nuccitelli, 1993; Miller et al., 1993). BAPTA and related compounds are thought to increase the rate of diffusion of calcium ions by shuttling them away from regions of high concentration, thereby dissipating Ca^{2+} gradients (Speksnijder et al., 1989). It is thought that the BAPTA buffer that is most effective at inhibiting a particular cellular function will have a K_D that is midway between the resting level and the peak of the Ca^{2+} gradient (Speksnijder et al., 1989).

Several studies (Han et al., 1992; Snow and Nuccitelli, 1993; Miller et al., 1993) have shown that dibromo-BAPTA (K_D for $Ca^{2+} \sim 1.5$ μM) is the most effective of the BAPTA buffers at inhibiting cleavage in *Xenopus* embryos, implying that gradients of calcium with peak concentrations of several micromolar are necessary for cell division. Likewise, Sullivan et al. (1993) report that fusion of nuclear envelope vesicles was most effectively inhibited by dibromo-BAPTA in *Xenopus* egg extracts.

These results all suggest that there should be substantial increases in $[Ca^{2+}]_i$ at several points in the *Xenopus* cell cycle. Although our calibrations show the peak calcium concentration to be less than 600 nM, this is the average concentration over the whole embryo and does not rule out the possibility that there are subdomains within the embryo with much higher calcium levels. The Ca^{2+} gradients that have been proposed to drive cytokinesis (Miller et al., 1993) would have to be highly localized, since there are no Ca^{2+} increases seen during cleavage (this study; Kubota et al., 1993). Similarly, because no calcium increases are seen during the later stages of mitosis

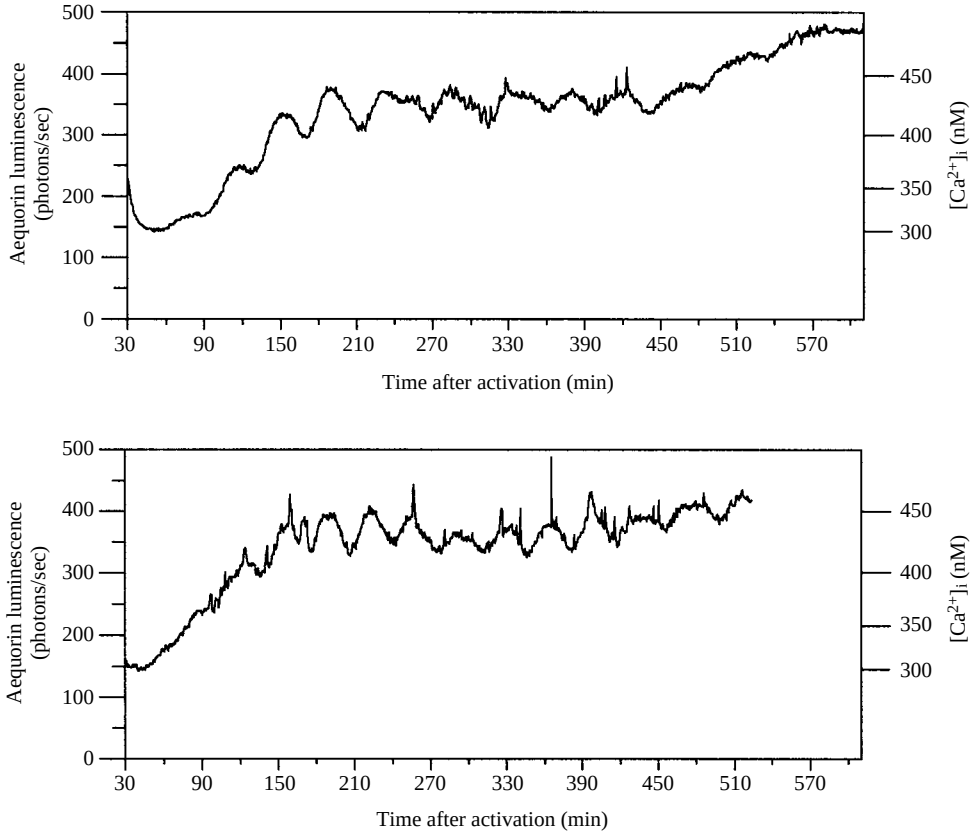

Fig. 5. Two examples of aequorin luminescence in artificially activated eggs. Unfertilized eggs were prick-activated by the aequorin injection. Shown are two records taken during the first 8-10 hours following activation. The calcium oscillations are similar to those measured in normal, fertilized eggs. The secondary pattern upon which the oscillations are superimposed rises during the first three cycles and stays elevated for the rest of the recording.

(this study; Kubota et al., 1993), the calcium gradients that are thought to be required for nuclear vesicle assembly (Sullivan et al., 1993) might be too brief and too spatially restricted to generate a signal above the noise in aequorin recordings.

Relationship between [Ca^{2+}]$_i$ oscillations and cell division

When we estimated the time of mitosis and cleavage we focused on the first three cleavages, since the blastomeres divide synchronously at this stage (Satoh, 1977). Mitosis began several minutes after the peak calcium level and was complete within about ten minutes, before calcium fell to its lowest level. Cleavage began in the animal hemisphere several minutes after the end of mitosis, at the beginning of the rising phase of the next calcium oscillation.

Source of [Ca^{2+}]$_i$ oscillations

The finding that the calcium oscillations continued in a normal manner in embryos that were bathed in calcium-free medium demonstrates that the calcium arises from intracellular sources. As it has been reported that *Xenopus* embryos do not have ryanodine-sensitive calcium stores (Galione et al., 1993), it seems likely that the major route for intracellular calcium release is via IP_3-sensitive stores (Berridge, 1993). Measurements of IP_3 mass in cleaving *Xenopus* embryos (Stith et al.,

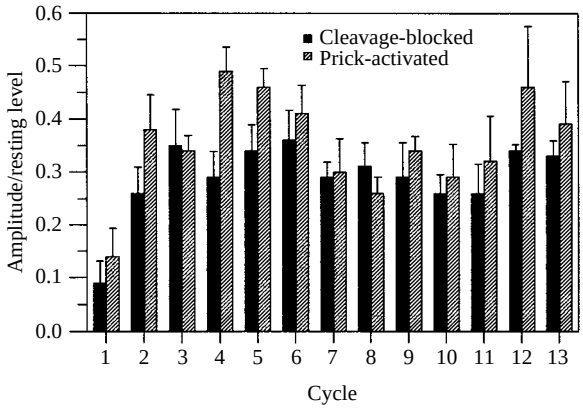

Fig. 6. Average peak-to-peak amplitudes of [Ca^{2+}]$_i$ oscillations in embryos treated with colchicine or nocodazole (filled bars) or prick-activated eggs (hatched bars) for the first 13 cycles following fertilization or activation. Since these embryos and eggs did not divide, the first Ca^{2+} cycle was taken to be the oscillation that peaked 60-90 minutes after fertilization or activation. The amplitudes were divided by the resting level of light output to correct for differences in the amount of active aequorin in each embryo or egg. Error bars indicate s.e.m.

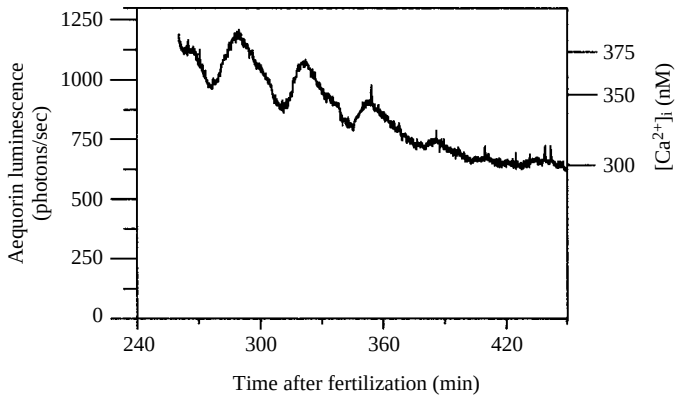

Fig. 7. Light output from an embryo in which aequorin was injected into a single 64-cell stage blastomere. The calcium signal from a subregion of the embryo is significantly different from the recordings from whole embryos at the same stage. The recording begins during mitosis of seventh cleavage.

1993) show that IP_3 levels transiently increase at about the time of mitosis, which is in general agreement with the elevations in calcium that we and others (Grandin and Charbonneau, 1991; Kubota et al., 1993) have measured at that point. That increases in IP_3 levels might be required for cell cycle progression is also supported by the work of Han et al. (1992), who showed that injection of heparin or antibodies to PIP_2 slows or stops cleavage. Both treatments inhibit IP_3 signaling and hence Ca^{2+} increases, heparin by competing with IP_3 for binding to its receptor and antibodies to PIP_2 by preventing production of IP_3.

Measurement of IP_3 levels in *Xenopus* embryos through the 2000-cell stage shows that IP_3 levels are low during the first few cleavages, increase and peak at the 64-cell stage, and thereafter slowly decline (Maslanski et al., 1992). This pattern of IP_3 variations correlates roughly with the secondary pattern of calcium changes underlying the oscillations that we and Kubota et al. (1993) have observed in dividing embryos. Whether changes in IP_3 levels can account for the changing baseline Ca^{2+} level and the varying amplitude of the oscillations is not known.

Spatial distribution of the $[Ca^{2+}]_i$ increases

We recorded light output from embryos that had been injected with aequorin into single 64-cell stage blastomeres. The signals from such embryos are distinctly different from those obtained from whole embryos at similar stages (compare Fig. 8A with Fig. 8C). While the Ca^{2+} oscillations in a whole embryo are roughly symmetrical, rising and falling at the same rates, the oscillations from a small subregion of the embryo rise more quickly and have a slower, biphasic decline (Fig. 8A). This indicates that the Ca^{2+} oscillations are not occurring uniformly within the multicellular embryo and that the signal we record from the whole embryo is the average of an array of localized, asynchronous calcium changes.

When the trace from Fig. 8A is averaged together with the same data shifted by 4, 8 and 12 minutes the resulting composite signal (Fig. 8B) closely resembles the whole-embryo recordings at later cleavages (Fig. 8C). This supports the idea that the whole-embryo recordings could be the average

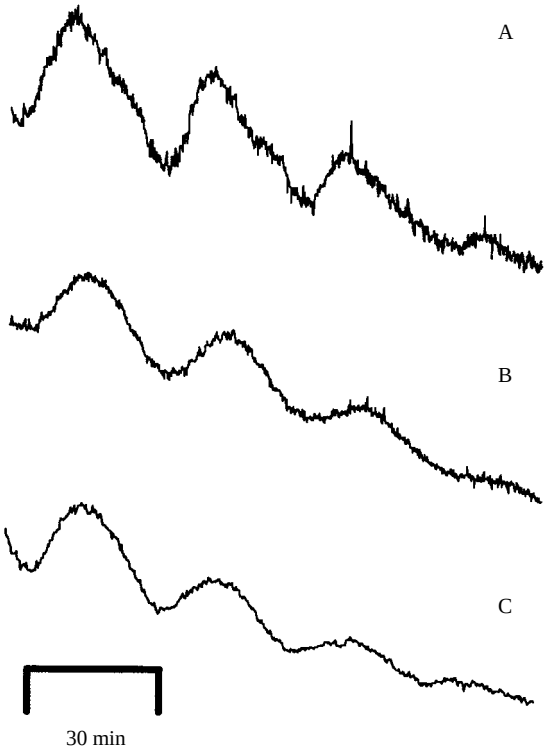

Fig. 8. (A-C) (A) A portion of the trace from Fig. 7, beginning at seventh cleavage, enlarged to show the shape of the calcium oscillations. (B) A composite signal constucted by averaging the data from A together with the same trace shifted by 4, 8 and 12 minutes. (C) A portion of the Ca^{2+} signal from Fig. 1A, beginning at the sixth cleavage. The shape of the calcium oscillations in A is different from those in the whole embryo recordings (C). The similarity between B and C indicates that the whole-embryo calcium signals could arise as the average of a collection of asynchronous regional recordings like those in A.

of a collection of localized signals, such as those in Fig. 7, that are out of phase with one another.

The amplitude of the oscillations was reduced as a result of this averaging of out of phase aequorin signals (Fig. 8B). In Fig. 8A the largest calcium change, from the peak of the first oscillation to the following trough, was 50 nM. After averaging the out of phase signals, the equivalent calcium change was only 30 nM (Fig. 8B). The amplitudes of the oscillations in whole-embryo recordings therefore represent lower limits of the Ca^{2+} increases, with the actual calcium concentrations depending on the extent to which the changes are localized.

Beginning at fifth cleavage the blastomeres of *Xenopus* embryos divide asynchronously, with each round of cleavage proceeding as a wave from the animal to the vegetal pole (Satoh, 1977). If the calcium oscillations are in phase with the cell cycle of each blastomere then the whole-embryo calcium recordings might be the average of the Ca^{2+} signals from a group of blastomeres whose cell cycles are up to 12 minutes out of phase with one another. In this case calcium oscillations would proceed as waves, since each round of cleavage occurs as a wave, although Ca^{2+} would be rising and falling uniformly within each blastomere.

If there are Ca^{2+} changes within each blastomere that

resemble those in Fig. 7, then one would expect to measure biphasic calcium oscillations during the first through fourth cleavage when the blastomeres' cell cycles are synchronous. The fact that the Ca^{2+} oscillations have roughly the same shape during the early synchronous cleavages and the later asynchronous divisions suggests that the calcium changes occur in a localized pattern during every round of cleavage. The calcium oscillations may spread across the embryo as waves, even during the early cleavages, perhaps in conjunction with surface contraction waves (Hara et al., 1980). At present, there is no evidence that there are calcium waves in cleaving embryos, although the recordings from subregions of the embryos are consistent with this model.

Kubota et al. (1993) attempted to determine whether there are localized Ca^{2+} changes in cleaving embryos using a photon-counting image processor. However, they were unable to draw any conclusions about this issue, perhaps because of a low signal to noise ratio (Kubota et al., 1993).

Careful mapping of the spatial distribution of the $[Ca^{2+}]_i$ changes will be useful in determining the functional role of the Ca^{2+} oscillations. Further improvements could come from imaging of $[Ca^{2+}]_i$ with fluorescent indicators, although these are not as sensitive as aequorin and the concentration of fura-2 required to obtain an adequate signal on our imaging system (100-200 μM) often inhibits cleavage. This latter problem is consistent with the observation that difluoro-BAPTA (K_D for Ca^{2+} ~ 0.25 μM; Snow and Nuccitelli, 1993), which has a similar affinity for Ca^{2+} to that of fura-2 (K_D for Ca^{2+} ~ 0.45 μM; Sullivan et al., 1993), inhibits cleavage when injected to a final concentration of ~150 μM (Snow and Nuccitelli, 1993). The fact that fluorescent Ca^{2+} indicators can interfere with cellular physiology in *Xenopus* eggs is also indicated by the observation that the fertilization $[Ca^{2+}]_i$ wave travels twice as fast in eggs containing 50 μM fura-2 compared to eggs loaded with 100 μM fura-2; the calcium wave is completely blocked in eggs containing 250 μM fura-2 (Nuccitelli et al., 1993).

Conclusions

By monitoring intracellular calcium levels with the luminescent protein aequorin, we have shown that there are oscillations of $[Ca^{2+}]_i$ in cleaving *Xenopus* embryos. The oscillations persist in embryos when cleavage is blocked by treatment with microtubule-disrupting drugs and are also present in artificially activated eggs. The increases in calcium are a result of calcium release from intracellular stores, since the oscillations still occur when the recordings are performed in calcium-free solution. The signal from the whole embryo differs from the signal obtained from a small subregion of the embryo, demonstrating that calcium levels are not uniform throughout the multicellular embryo and suggesting that the Ca^{2+} increases might cross the embryos as waves.

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