

# Intracellular Free Calcium Oscillates during Cell Division of *Xenopus* Embryos

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**Abstract.** In *Xenopus* embryos, previous results failed to detect changes in the activity of free calcium ions ( $\text{Ca}^{2+}$ ) during cell division using  $\text{Ca}^{2+}$ -selective microelectrodes, while experiments with aequorin yielded uncertain results complicated by the variation during cell division of the aequorin concentration to cell volume ratio. We now report, using  $\text{Ca}^{2+}$ -selective microelectrodes, that cell division in *Xenopus* embryos is accompanied by periodic oscillations of the  $\text{Ca}^{2+}$  level, which occur with a periodicity of 30 min, equal to that of the cell cycle. These  $\text{Ca}^{2+}$  oscillations were

detected in 24 out of 35 experiments, and had a mean amplitude of 70 nM, around a basal  $\text{Ca}^{2+}$  level of 0.40  $\mu\text{M}$ .  $\text{Ca}^{2+}$  oscillations did not take place in the absence of cell division, either in artificially activated eggs or in cleavage-blocked embryos. Therefore,  $\text{Ca}^{2+}$  oscillations do not represent, unlike intracellular pH oscillations (Grandin, N., and M. Charbonneau. *J. Cell Biol.* 111:523–532. 1990), a component of the basic cell cycle (“cytoplasmic clock” or “master oscillator”), but appear to be more likely related to some events of mitosis.

**D**URING the past few years, cell division of early embryos has proved to be an interesting model system to study the mechanisms of the basic cell cycle governing mitosis. There is accumulating evidence that one of the molecular components of this basic cycle, or “master oscillator,” is represented by a universal M-phase promoting factor (MPF)<sup>1</sup> (Masui and Markert, 1971) and its correlated *cdc2* kinase activity. Indeed, one of the components of MPF, a 34-kD protein, is homologous to the product of the *cdc2* gene in *Saccharomyces pombe*, in frog eggs (Dunphy et al., 1988; Gautier et al., 1988) and starfish oocytes (Arion et al., 1988; Labbé et al., 1988, 1989b). In addition, a second component of MPF has been identified as cyclin in starfish oocytes, sea urchin eggs, and *Xenopus* eggs (Labbé et al., 1989a; Meijer et al., 1989; Gautier et al., 1990). The egg of *Xenopus laevis* has now become one of the most important systems for studying the molecular biology of the cell division cycle. Considerable information about MPF, cyclins, and control of both meiosis and mitosis has been uncovered using the *Xenopus* system. However, almost no attention has been given to the possible involvement of ionic messengers, particularly intracellular free calcium ( $\text{Ca}^{2+}$ ), in the control of mitosis in *Xenopus* embryos.

A variety of different systems has revealed direct implication of  $\text{Ca}^{2+}$  in the onset of mitosis, the metaphase-anaphase transition, and exit from mitosis (reviewed by Berridge and Irvine, 1989; Hepler, 1989). Variations in  $\text{Ca}^{2+}$  at specific mitotic stages might act through effects on various cell cycle proteins (reviewed by Whitaker and Patel, 1990).

To date, in the few previously published attempts, no such variations in  $\text{Ca}^{2+}$  have been detected in embryos of *Xenopus*. Thus, using  $\text{Ca}^{2+}$ -selective microelectrodes, neither Rink et al. (1980), nor Busa and Nuccitelli (1985) were able to measure  $\text{Ca}^{2+}$  variations during the *Xenopus* early cell cycle, while Baker and Warner (1972), using the aequorin luminescence technique, reported variations during cell division, but were confronted with the problem of the variation of the aequorin concentration to cell volume ratio as blastomeres became smaller and smaller.

The probable role of  $\text{Ca}^{2+}$  in the control of mitosis in various systems, as well as the strong intuition we had that *Xenopus* embryos did display  $\text{Ca}^{2+}$  variations, led us to reinvestigate the situation. We also chose the  $\text{Ca}^{2+}$ -selective microelectrode technique. First, this technique avoids the problem of the variation of the fluorescent probe concentration as cell volume changes during cell division. In addition, the fluorescent probes, such as fura-2 or quin-2, have the disadvantage of somewhat buffering the free  $\text{Ca}^{2+}$  that is being measured.  $\text{Ca}^{2+}$ -selective microelectrodes do not buffer the free  $\text{Ca}^{2+}$  within the cell, while measuring submicromolar levels of  $\text{Ca}^{2+}$  in living cells with a response time of a few seconds only. In the present study, we report that cell division of *Xenopus* embryos is accompanied by periodic oscillations of the  $\text{Ca}^{2+}$  level (amplitude: 70 nM) around a basal  $\text{Ca}^{2+}$  level of 0.40  $\mu\text{M}$ . These  $\text{Ca}^{2+}$  oscillations occurred with a periodicity of 30 min, equal to that of the *Xenopus* embryonic cell cycle. In addition,  $\text{Ca}^{2+}$  oscillations were detected only in association with cell division, but not in artificially activated (nondividing) eggs or in cleavage-blocked embryos, suggesting a role for these  $\text{Ca}^{2+}$  oscilla-

1. Abbreviation used in this paper: MPF, M phase promoting factor.

tions in the control of cell division. These findings are compared to those obtained from other model systems, and discussed in relation to the presence of other cyclic activities during the early cell cycle of *Xenopus* embryos.

## Materials and Methods

### Animals and Solutions

Mature females of *Xenopus*, reared in the laboratory, were induced to ovulate by injection of 900 i.u. of human chorionic gonadotropin (hCG). Mature oviposited eggs were dejellied with 2% cysteine in F1 solution, modified from Hollinger and Corton (1980), which contained (in mM): 31.2 NaCl, 1.8 KCl, 1.0 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 1.9 NaOH, and 2.0 NaHCO<sub>3</sub>; buffered at pH 7.4–7.5 with 10 mM Hepes.

Mature eggs were inseminated with a sperm suspension obtained by crushing a piece of testis in F1 solution. Around 10 min later, eggs were dejellied and impaled with microelectrodes (see below). Cell division was visually recorded under a stereomicroscope during electrical recording.

Nocodazole (methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate), an inhibitor of microtubule assembly (De Brabender et al., 1976) was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared in DMSO-ethanol (vol/vol) as a stock solution of 1 mg/ml.

### Intracellular Free Ca<sup>2+</sup> (Ca<sup>2+</sup>i) Measurements

Intracellular Ca<sup>2+</sup>-selective microelectrodes were fabricated as described previously (Tsien and Rink, 1980; Busa and Nuccitelli, 1985; Busa, 1986). Glass capillaries without an inner fiber (GC 150; Clark Electromedical Instruments, Pangbourne, Reading, England) were drawn on a Campden micropipette puller. Micropipettes were broken at their tips to ~2 μm and rendered hydrophobic with tributylchlorosilane, after baking for 2 h at 180°C. The micropipettes were then backfilled via pressure with pCa 7 calibration buffer (see below), and their tips filled by suction with a 20–100-μm column of a ready-to-use Ca<sup>2+</sup> sensor (calcium ionophore I, Cocktail A; Fluka Chemical Corp., Buchs, Switzerland) designed by Lanter et al. (1982). Ca<sup>2+</sup> microelectrodes were calibrated in pCa 6 and pCa 7 solutions containing, respectively, 10.0 mM EGTA, 5.0 mM CaCl<sub>2</sub>, 10.0 mM Pipes, 45.0 mM KOH, 15.0 mM KCl, pH 6.77 (at 23°C), and 10.0 mM EGTA, 5.0 mM CaCl<sub>2</sub>, 10.0 mM MOPS, 35.5 mM KOH, 29.3 mM KCl, pH 7.27 (at 23°C), as described in Busa and Nuccitelli (1985). The Ca<sup>2+</sup> response of these microelectrodes was 24–34 mV between pCa 6 and pCa 7, with a full response time of a few seconds. Unactivated eggs were impaled with microelectrodes without recourse to any anesthetic. Dejellied embryos were implanted with microelectrodes 20–30 min after fertilization. Each unactivated egg or embryo, immersed in F1 solution in the recording chamber, a 4-ml tissue culture plastic dish (60 × 15 mm) with a center well (Falcon Labware, Oxnard, CA), was impaled with a potential microelectrode (GC 150F capillaries with an inner fiber), filled with 3 M KCl, 10 mM EDTA, and 10 mM potassium citrate, and a Ca<sup>2+</sup> microelectrode. Membrane potential (Em) was subtracted, at the pen recorder input, from the total signal recorded by the ion-specific microelectrode, which corresponded to the ionic activity measured (pCa, the negative log of free Ca<sup>2+</sup> activity) plus the membrane potential, Em. Membrane potentials and ion-specific signals were recorded by high input amplifiers (Burr Brown OPA 104, Le Chesnay, France) and connected to the ground via an F1 agarose bridge. Other details for electrical recordings have been described elsewhere (Grandin and Charbonneau, 1989).

### Intracellular Free Mg<sup>2+</sup> (Mg<sup>2+</sup>i) Measurements

Intracellular Mg<sup>2+</sup>-selective microelectrodes were fabricated according to the same procedure as that used to make Ca<sup>2+</sup>-selective microelectrodes, except that their tips were filled with a 20–100-μm column of a ready-to-use Mg<sup>2+</sup> sensor (magnesium ionophore, Cocktail A; Fluka Chemical Corp.). The resin-filled microelectrodes were backfilled with 10 mM MgCl<sub>2</sub> and calibrated in pure MgCl<sub>2</sub> solutions and in solutions containing 0.05–50 mM free Mg<sup>2+</sup> and KCl, as described previously (Sui and Shen, 1986). Potassium chloride was added to the calibrating solutions in order to account for the known interference of Mg<sup>2+</sup> microelectrodes with K<sup>+</sup> ions (see references in Sui and Shen, 1986). We used 90 mM KCl in the calibrating solutions, instead of the 220 mM employed in the study by Sui and Shen (1986) on sea urchin eggs, because 90 mM represent the measured internal

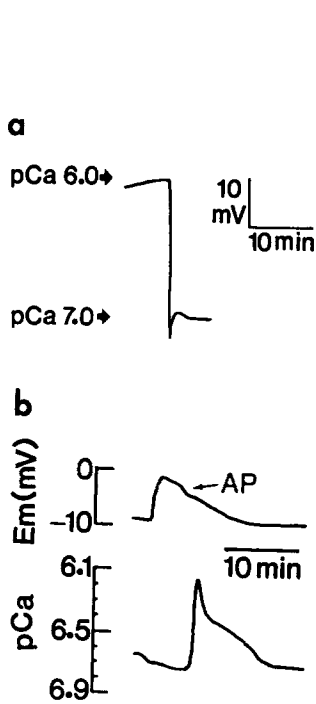
K<sup>+</sup> concentration in amphibian eggs (Rodeau and Vilain, 1987). The slope of the response was 20–28 mV/decade, between 0.05 and 50 mM free Mg<sup>2+</sup>, in pure MgCl<sub>2</sub> solutions, and 18–22 mV/decade between 0.5 and 50 mM free Mg<sup>2+</sup>, and 10–13 mV/decade between 0.05 and 0.5 mM free Mg<sup>2+</sup>, when the calibrating MgCl<sub>2</sub> solutions were supplemented with 90 mM KCl. Thus, the reduction in slope in the presence of K<sup>+</sup>, which was very marked only between 0.05 and 0.5 mM free Mg<sup>2+</sup>, was not as evident as in the study by Sui and Shen (1986), which may be due to the lower KCl concentration employed here. In addition, we observed a shifting of the calibration baseline (variable from one microelectrode to the other) when replacing a K<sup>+</sup>-free calibrating solution by one containing 90 mM KCl and the same amount of Mg<sup>2+</sup>. Consequently, Mg<sup>2+</sup> ion activities inside the embryos were calculated using the calibration traces obtained in KCl-containing solutions, and expressed as pMg (the negative log of free Mg<sup>2+</sup> activity). Contrary to Sui and Shen (1986), we never observed a shifting of the calibration baseline after impalement of the egg, even after our longer experiments (4 h recording).

## Results

In our current study, Ca<sup>2+</sup> microelectrodes are used to measure the variations in Ca<sup>2+</sup>i during *Xenopus* egg activation. They have been found to constantly detect Ca<sup>2+</sup>i transients with characteristics exactly similar to those previously described (Busa and Nuccitelli, 1985). Measuring the large spike at egg activation served as a test for our detection system (Fig. 1). Such recordings demonstrated that our Ca<sup>2+</sup> microelectrodes were sensitive to submicromolar variations of the Ca<sup>2+</sup>i level, and that, in addition, they had a short full response time (of the order of a few seconds) and were selective for Ca<sup>2+</sup> ions.

Fig. 2 shows typical Ca<sup>2+</sup>i variations during cell division in early embryos of *Xenopus*. When detected, which was the case in 24 out of 35 experiments, these Ca<sup>2+</sup>i oscillations were found to occur cyclically, with a periodicity equal to that of the cell cycle (29.4 ± 2.9 min, mean value ± SD, n = 53, in 24 embryos from 14 females, at 22–24°C). Ca<sup>2+</sup>i oscillations had a mean amplitude, peak to peak, of 0.07 ± 0.03 μM (SD, n = 75, 24 embryos from 14 females), and oscillated around a basal Ca<sup>2+</sup>i level of 0.41 ± 0.16 μM (n = 75). In the experiment shown in Fig. 2, Ca<sup>2+</sup>i did not begin to oscillate until the 64-cell stage. However, this was not the case in all experiments. In 11 out of the 24 experiments in which they were detected, the Ca<sup>2+</sup>i oscillations appeared as early as the two-, four-, or eight-cell stage (Fig. 3 a).

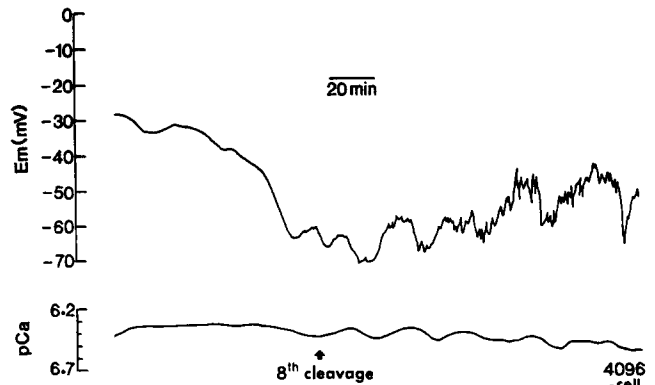
Two sorts of control experiments were conducted in order to rule out the possible intervention of artifacts in the generation of these Ca<sup>2+</sup>i oscillations. First, we had to verify that contractions or cell surface movements associated with the cleavage of blastomeres, and therefore synchronous with the cell cycle, were not generating so called "motion artifacts." Indeed, due to the very high impedance of ion-selective microelectrodes, such contractions or movements might create artifactual electrical signals resulting from a mechanical pressure on the microelectrode tip. To rule out such a possibility, we used a simple approach which consisted of impaling embryos with Mg<sup>2+</sup>-selective microelectrodes, which have the same impedance as Ca<sup>2+</sup> microelectrodes. In none of the 14 embryos in which Mg<sup>2+</sup> activity was measured for 3–4 h, starting at the two-cell stage, could we detect oscillations of the internal Mg<sup>2+</sup> activity. In addition, embryos which were impaled simultaneously with a Ca<sup>2+</sup> microelectrode and a Mg<sup>2+</sup> microelectrode (and two potential micro-



**Figure 1.** (a) Calibration traces of a  $\text{Ca}^{2+}$ -selective microelectrode at the indicated pCa levels (negative log of free  $\text{Ca}^{2+}$  activity). Only those microelectrodes with a response slope ranging from 24 to 34 mV between pCa 6 and pCa 7 were used for  $\text{Ca}^{2+}$ i measurements. (b) A typical example of  $\text{Ca}^{2+}$ i transient triggered upon egg activation, in *Xenopus laevis*, with characteristics similar to those previously described (Busa and Nuccitelli, 1985). Such recordings served as a test for the sensitivity, specificity, and rapidity of our  $\text{Ca}^{2+}$  microelectrodes.  $\text{Ca}^{2+}$ i transiently increased soon after activation (detected by the occurrence of an activation potential (top trace) and returned within the next 10 min to the same resting level as before activation.

electrodes) and displayed  $\text{Ca}^{2+}$ i oscillations, did not display any  $\text{Mg}^{2+}$ i oscillation (Fig. 4 a). A second sort of artifact might have been introduced by the subtraction of the membrane potential from the total signal recorded by the  $\text{Ca}^{2+}$ i microelectrode (see Materials and Methods), generating so called "mirror image" artifacts. If the membrane potential was slightly different between the two impaled blastomeres, because of electrical uncoupling between these two blastomeres or because of the incorrect insertion of one of the microelectrodes, then a variation in the  $\text{Ca}^{2+}$  microelectrode output would ensue, that would be in fact a membrane potential change in only one of these two blastomeres. Several observations and controls argue against an artifact introduced by incorrect subtraction of the membrane potential from the  $\text{Ca}^{2+}$  microelectrode output (see Discussion). Additional evidence was provided by experiments in which embryos were impaled simultaneously with four potential microelectrodes, starting at the two-cell stage. In all five long term recording experiments (3–5 h), the membrane potential was found to be exactly the same in the four blastomeres located respectively in each of the four animal quarters of the embryo (Fig. 4 b). In all 12 other experiments performed with two potential microelectrodes impaled in the same embryo, the membrane potential remained the same in two blastomeres located on opposite regions of the animal hemisphere during the 3–4 h of recording. These experiments demonstrate that blastomeres of early *Xenopus* embryos remain electrically coupled during at least the 5 h after the first cell division.

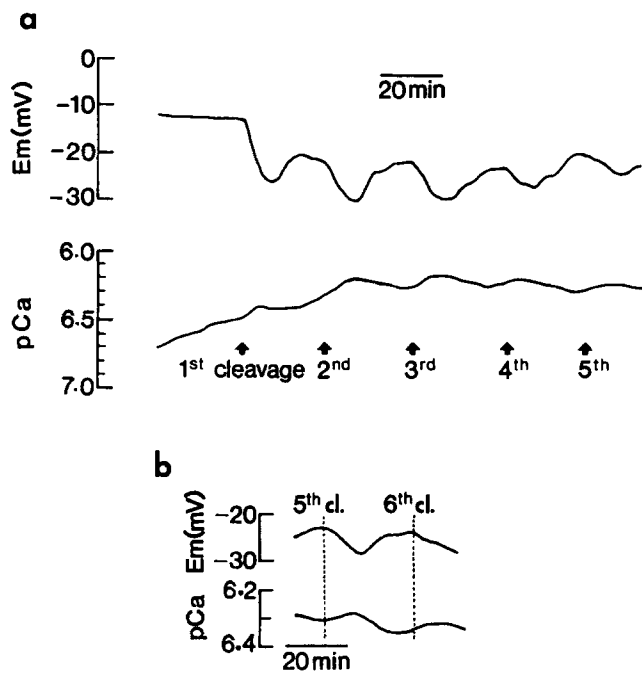
Interestingly, the peak level of the  $\text{Ca}^{2+}$ i oscillations occurred only a few minutes after the beginning of the membrane hyperpolarizations which accompany cell division in *Xenopus* embryos (Fig. 3 a). In *Xenopus* embryos, each cleavage is associated with a membrane hyperpolarization which corresponds to the fabrication of new plasma membrane in the forming blastomeres (Woodward, 1968; De



**Figure 2.**  $\text{Ca}^{2+}$ i oscillations in embryos of *Xenopus laevis*, measured with  $\text{Ca}^{2+}$  microelectrodes. This is an example of  $\text{Ca}^{2+}$ i cycling during the cell division cycle. Such long recordings were difficult to obtain, for technical reasons. Indeed, as cell division proceeds, the  $\text{Ca}^{2+}$  microelectrode (bottom trace) and the potential microelectrode (top trace), which is also necessary for  $\text{Ca}^{2+}$ i measurement (see Materials and Methods), become located in two different blastomeres. If the membrane potential is not exactly the same in these two blastomeres, because of the incorrect insertion of one of the microelectrodes, this generates a so-called "mirror image." In addition, the blastomeres of the dividing embryo become smaller and smaller; thus, a microelectrode becomes frequently located between two blastomeres after the blastomere in which it was previously impaled has divided. Finally, it frequently happens that the microelectrodes actually come out of these small blastomeres, possibly due to reorganizations of some cytoskeletal components. In this particular example,  $\text{Ca}^{2+}$ i oscillations were recorded only after the 64-cell stage. This was also the case in 12 other experiments. The period of the  $\text{Ca}^{2+}$ i oscillations was equal to that of the cell cycle, 30 min in the present experiment. This embryo continued to cleave normally and  $\text{Ca}^{2+}$ i to oscillate until the 4096-cell stage, the end of the experiment.

Laat and Bluemink, 1974). The beginning of each of the hyperpolarizing phases corresponds to the onset of cleavage (cytodieresis), while the end of each of these phases marks the completion of cleavage, as observed under a stereomicroscope on embryos impaled with microelectrodes. We could observe that the peak level of the  $\text{Ca}^{2+}$ i oscillation occurred 10–20 min after the onset of cleavage, indicated by membrane potential hyperpolarization (Fig. 3 b).

Another finding of the present work is that the  $\text{Ca}^{2+}$ i oscillations were recorded only in association with cell division. Indeed, in all 16 long term (at least 6-h recording after activation) experiments performed, we could not detect any  $\text{Ca}^{2+}$ i oscillations in eggs which were artificially activated by pricking, and therefore did not divide (Fig. 5 a). With the exception of cell division, artificially activated eggs undergo almost all of the metabolic events characterizing fertilized eggs, including surface contraction waves (Hara et al., 1980), cycling of MPF activity (Dabauvalle et al., 1988), and pHi oscillations (Grandin and Charbonneau, 1990). These latter activities are components of the basic cell cycle, occurring independently of cell division. We show here that, on the contrary,  $\text{Ca}^{2+}$ i oscillations do not seem to occur in the absence of cell division. However, since  $\text{Ca}^{2+}$ i oscillations were not always detected during the initial cell divisions (see Fig. 2), it was possible that the  $\text{Ca}^{2+}$  microelectrodes could detect oscillations only in a restricted cell



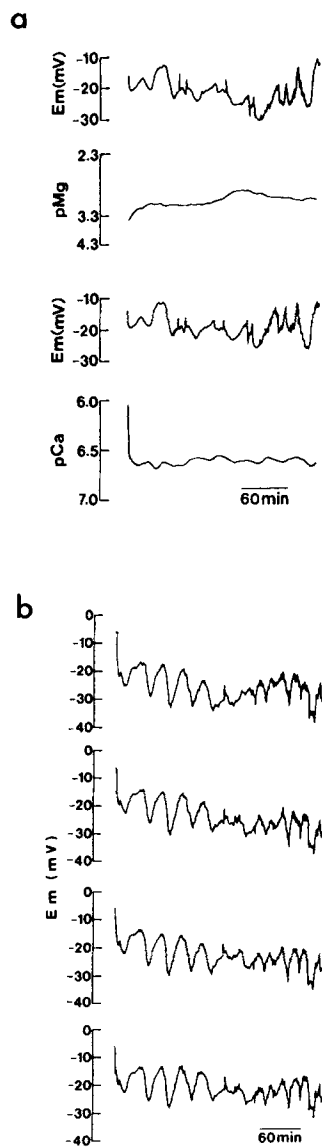
**Figure 3.** (a) Example of  $\text{Ca}^{2+}_i$  measurement in an embryo of *Xenopus* illustrating the fact that  $\text{Ca}^{2+}_i$  oscillations occur as early as the first cell division. This trace also shows that the peak level of the  $\text{Ca}^{2+}_i$  oscillation actually followed the hyperpolarizing phase, indicating the onset of cleavage, by a few minutes. (b) Difference of phase between membrane potential cycling and  $\text{Ca}^{2+}_i$  cycling. In this and all  $\text{Ca}^{2+}_i$  and potential recordings, the peak level was found to occur 10–20 min after the onset of cleavage (see text).

volume due to local  $\text{Ca}^{2+}_i$  variations, a situation favored when recording is performed in very small blastomeres. In other words, it was possible that artificially activated eggs did display  $\text{Ca}^{2+}_i$  oscillations, which could not be detected because of the positioning of the microelectrodes out of the region concerned with  $\text{Ca}^{2+}_i$  cycling. Consequently, in order to solve this problem, embryos displaying  $\text{Ca}^{2+}_i$  oscillations were treated with nocodazole (an inhibitor of microtubule assembly). An arrest of  $\text{Ca}^{2+}_i$  cycling was noted in correlation with the arrest of cell division by nocodazole (Fig. 5 b). It appears therefore that the cyclic variations in  $\text{Ca}^{2+}_i$  during cell division of *Xenopus* embryos, described here for the first time, might be correlated to specific stages of mitosis.

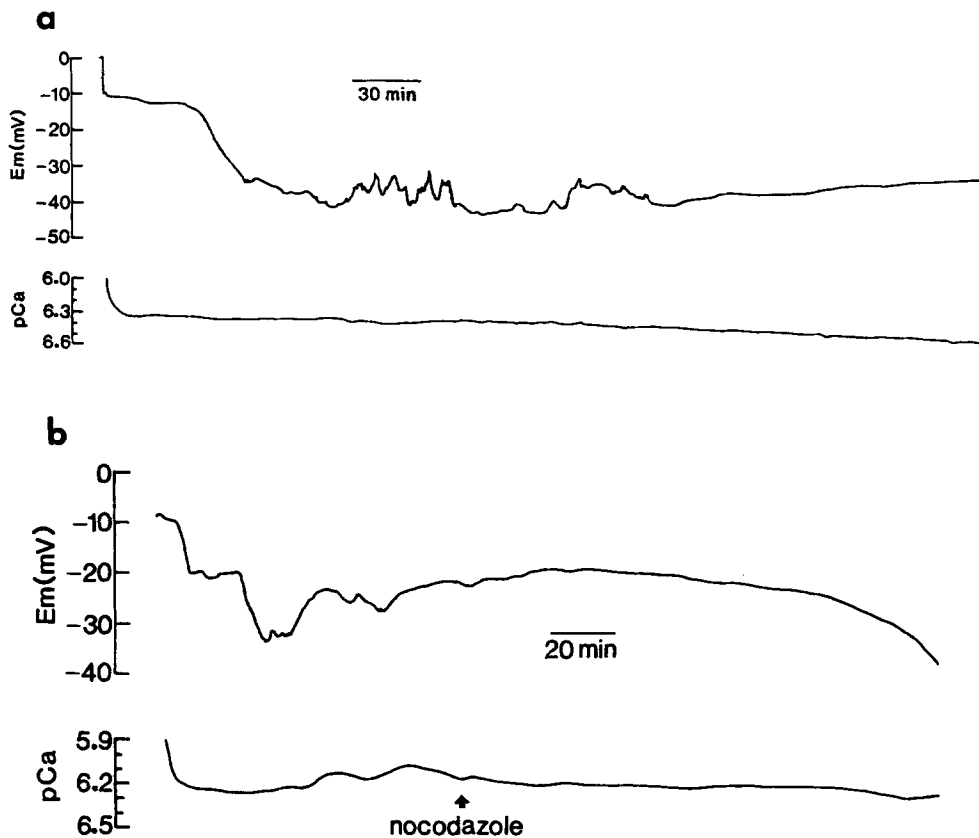
## Discussion

These first measurements of  $\text{Ca}^{2+}_i$  variations during cell division of *Xenopus* embryos indicate that this species does not represent an exception, contrary to what has been frequently written during the past few years.

Superimposed on the constant basal  $\text{Ca}^{2+}_i$  level of  $0.40 \mu\text{M}$  are small oscillations that average  $70 \text{ nM}$  and have a period exactly coincident to that of the cell division cycle, that is  $\sim 30 \text{ min}$ . Several investigators have tried previously to detect  $\text{Ca}^{2+}_i$  variations during the *Xenopus* early cell cycle, without success. The first attempt was made by injecting the  $\text{Ca}^{2+}$ -sensitive protein aequorin into *Xenopus* embryos (Baker and Warner, 1972). Five among the eight injected embryos displayed an increase in light output at first and sec-



**Figure 4.** (a) Control experiments using  $\text{Mg}^{2+}$ -selective microelectrodes. This embryo was impaled at the two-cell stage with a  $\text{Mg}^{2+}$  microelectrode, a  $\text{Ca}^{2+}$  microelectrode, and two potential microelectrodes. Each trace of ion activity measurement has its corresponding membrane potential trace (the one subtracted from the total signal recording by the ion-selective microelectrode) represented above it. Soon after impalement,  $\text{Ca}^{2+}_i$  started to oscillate around its basal level,  $0.24 \mu\text{M}$ , during six cell division cycles. Meanwhile,  $\text{Mg}^{2+}_i$ , recorded simultaneously in the same embryo, displayed a nonoscillating level. The presence of  $\text{Ca}^{2+}_i$  oscillations was found to be associated with an absence of  $\text{Mg}^{2+}_i$  oscillations in the same embryo in two other experiments (2–3 h recording). In the example shown here,  $\text{Mg}^{2+}_i$ , after stabilization following impalement, was  $0.9 \text{ mM}$ . The reduction in the slope of the  $\text{Mg}^{2+}$  microelectrodes between  $0.05 \text{ mM}$  free  $\text{Mg}^{2+}$  ( $\text{pMg } 4.3$ ) and  $0.5 \text{ mM}$  free  $\text{Mg}^{2+}$  ( $\text{pMg } 3.3$ ) in the presence of  $90 \text{ mM}$  KCl in the calibration solutions, as explained in Materials and Methods, is well visible from the ion activity scale. In all 14 other experiments in which  $\text{Mg}^{2+}_i$  was recorded for 3 to 4 h, starting at the two-cell stage, we also noted an absence of  $\text{Mg}^{2+}_i$  oscillations. The mean value of internal  $\text{Mg}^{2+}$  activity in *Xenopus* embryos (stabilized level at the two- or four-cell stage) was  $1.3 \pm 0.5 \text{ mM}$  (SD,  $n = 33$ ). 19 out of the 33 embryos in which  $\text{Mg}^{2+}_i$  was measured were not considered for assessing the presence or absence of  $\text{Mg}^{2+}_i$  oscillations because the microelectrodes remained correctly impaled for  $<3 \text{ h}$ . In this study, we could not discern any obvious pattern of  $\text{Mg}^{2+}_i$  variations associated with cell division.  $\text{Mg}^{2+}_i$  levels remained very stable, or, sometimes (as in the example shown here), displayed large and slow, but not reproducible, variations. (b) Control experiments with two or four potential microelectrodes impaled in a single embryo. This embryo was impaled at the two-cell stage with four potential microelectrodes in order to demonstrate correct electrical coupling between *Xenopus* blastomeres. At the end of the experiment, after  $\sim 10$  cell division cycles, the four microelectrodes were located each in one of the four animal quarters of the embryo, which probably contained  $\sim 2,000$  cells. During the entire course of recording, membrane potential remained the same in the different regions of the embryo. Such an electrical coupling, assessed by comparing membrane potential traces recorded simultaneously with four potential microelectrodes in well developing embryos, was observed in four other experiments (3–4 h recording). In addition, electrical coupling was also correct in all 12 other experiments in which the membrane potential was recorded simultaneously in two opposite regions of the animal hemisphere of the embryo (3–4 h recording).



**Figure 5.** (a) Long term recording of  $\text{Ca}^{2+}_i$  and membrane potential in an artificially activated egg. This egg was activated by pricking and impaled with microelectrodes 1 h 20 min later. Pricking of the cortex of *Xenopus* eggs is known to activate the eggs by allowing the entry of extracellular  $\text{Ca}^{2+}$  ions (Wolf, 1974). In this and 15 other experiments,  $\text{Ca}^{2+}_i$  did not change at all during several hours. (b) Disappearance of  $\text{Ca}^{2+}_i$  cycling after blockade of cell division with nocodazole (10  $\mu\text{g}/\text{ml}$ ). This egg was impaled with microelectrodes 1 h 40 min after insemination, soon before the onset of second cleavage, indicated by membrane hyperpolarization (top trace).  $\text{Ca}^{2+}_i$  began to oscillate at the eight-cell stage (bottom trace). Nocodazole, added at the 32-cell stage, produced an arrest of the  $\text{Ca}^{2+}_i$  oscillations, together with an arrest of cell division, because of its inhibitory action on spindle microtubule assembly. An arrest of  $\text{Ca}^{2+}_i$  oscillations by nocodazole was observed in four other experiments.

ond cleavages. However, that increase might have been due to a reduction of internal aequorin absorption during cleavage (Baker and Warner, 1972). The second attempt, reporting experiments that used  $\text{Ca}^{2+}$ -selective microelectrodes as in the present study, also failed to detect  $\text{Ca}^{2+}_i$  variations during *Xenopus* cell division (Rink et al., 1980). On first analysis, there is no difference between the technique used by these authors and that used in the present study, since they are both based on the technique described by Tsien and Rink (1980). There may be, however, slight modifications of fabrication, particularly concerning the omission or not of the step consisting of dipping the microelectrode tip into poly (vinyl chloride)-gelled  $\text{Ca}^{2+}$  sensor (see Tsien and Rink, 1981; Busa and Nuccitelli, 1985; Busa, 1986). After comparing both techniques, we chose to omit that step. Another difference between  $\text{Ca}^{2+}$ -selective microelectrodes fabricated at different times along the past decade may reside in the nature of the  $\text{Ca}^{2+}$  sensor used. For instance, neutral carrier-based  $\text{Ca}^{2+}$  microelectrodes with subnanomolar detection limit have recently been designed (Schefer et al., 1986; Ammann et al., 1987). We used calcium ionophore I, commercialized by Fluka Chemical Corp., and designed by Lanter et al. (1982), which may have been a little more selective and sensitive than the neutral carrier-based  $\text{Ca}^{2+}$  sensor used by Rink et al. (1980). The third attempt that was made to detect  $\text{Ca}^{2+}_i$  variation during *Xenopus* cell division also used  $\text{Ca}^{2+}$ -selective microelectrodes (Busa and Nuccitelli, 1985). Although these authors conducted a very care-

ful study on  $\text{Ca}^{2+}_i$  changes during *Xenopus* egg fertilization, they seemed to pay little attention to subsequent cleavage. Indeed, they report that four eggs failed to display  $\text{Ca}^{2+}_i$  changes during the first cleavage divisions (Busa and Nuccitelli, 1985). Analysis of the present results reveals that in only 11 embryos, out of the 24 in which  $\text{Ca}^{2+}_i$  oscillations were detected, were  $\text{Ca}^{2+}_i$  changes measured as early as the two-, four-, or eight-cell stage. This explains why Busa and Nuccitelli (1985) may have missed that event in very early embryos. For the moment, we have no explanation for the fact that  $\text{Ca}^{2+}_i$  oscillations were not detected in all cases in these early embryos. It might well be possible that, due to spatially restricted  $\text{Ca}^{2+}_i$  changes likely to exist within the cytoplasm,  $\text{Ca}^{2+}_i$  changes are more reliably detected with the  $\text{Ca}^{2+}$  microelectrodes during later cleavages than during the early cell divisions, due to the diminution of cell volume as cleavage proceeds.

The two main advantages of  $\text{Ca}^{2+}$ -selective microelectrodes, rapidity of the response (within 5–10 s) and absence of perturbation of the cell metabolism (the eggs cleave normally), are strengthened by the great selectivity and sensitivity that the new neutral carrier-based  $\text{Ca}^{2+}$  sensors, now commercially available (Fluka Chemical Corp.), allow. Therefore, the technology and methodology applied here to the embryos of *Xenopus laevis* appear to be adequate to detect  $\text{Ca}^{2+}_i$  variations. A very important precaution while measuring intracellular ion activities is to verify that the membrane potential measured by the ion-selective micro-

electrode is exactly the same as that measured by the potential microelectrode (see Materials and Methods). Indeed, in case it would not be the same, this would generate "mirror image" artifacts, due to the membrane potential being slightly different between these two blastomeres. Our control experiments which demonstrate that the membrane potential is the same in two opposite blastomeres, or in four distinctly located blastomeres, during at least the 5 h following the first cell division (see Fig. 4 b), rule out the possibility of partial uncoupling between blastomeres. When problems of membrane potential subtraction from the  $\text{Ca}^{2+}$  microelectrode output were encountered, they were either due to the fact that one of the microelectrodes had been incorrectly inserted, or, more frequently, to the fact that the microelectrodes were extruded from the impaled blastomeres during cleavage. Such an incorrect reading of the membrane potential by one of the two microelectrodes generates "mirror image" artifacts. However, provided one is cautious and careful, these technical difficulties are easily avoided, because the membrane potential mirror images are easily detected during the course of the experiment. None of our recordings selected for the presence of  $\text{Ca}^{2+}$  oscillations presented such a problem. Recordings in which we suspected, on the  $\text{Ca}^{2+}$  trace, a mirror image of the membrane potential recorded by the potential microelectrode were immediately discontinued. In addition, it should be noted that such mirror images would change the  $\text{Ca}^{2+}$  level by a nonnegligible value, which would have made our mean  $\text{Ca}^{2+}$  level values more scattered than they are. It is also clear from our recordings that very abrupt changes in membrane potential, associated with formation of new membrane during cleavage did not alter at all the basal  $\text{Ca}^{2+}$  level. Finally, it seems very unlikely that a possible mirror image artifact, which is random in nature, can mimic an almost perfect cyclical  $\text{Ca}^{2+}$  variation. This would be statistically impossible. This type of objection, that is suspicion of mirror images generated by electrodes being inserted in slightly uncoupled blastomeres, has already been ruled out in a similar situation. Indeed, Webb and Nuccitelli (1981) demonstrated the presence of intracellular pH (pHi) oscillations during cell division of *Xenopus* embryos. However, Lee and Steinhardt (1981) argued this pHi cycle, which they could record only in two experiments, was due to uncoupling between blastomeres, generating mirror images mimicking a natural cycle, as explained above. However, it became soon clear that this interpretation was wrong, since pHi cycling could be detected also when cleavage-associated membrane hyperpolarizations were prevented (Webb and Nuccitelli, 1982). We are particularly aware of this mirror image problem, and have also recently demonstrated the reality of pHi cycling in activated eggs and nondividing embryos of *Xenopus* (Grandin and Charbonneau, 1990). Accurate analysis of the traces obtained in the present study (24 displaying  $\text{Ca}^{2+}$  oscillations) clearly shows that the membrane potential variations associated with cleavage are not highly reproducible from one embryo to the other. For instance, in some cases, two membrane hyperpolarization phases appear within the same cycle instead of one as is the rule. Yet, in these cases, there is no corresponding mirror image displacement on the  $\text{Ca}^{2+}$  trace. The possibility that cell surface movements occurring in the cleaving blastomeres are responsible for the artifactual generation of  $\text{Ca}^{2+}$  oscillations ("motion artifacts") is strongly reduced by our

experiments using  $\text{Mg}^{2+}$  microelectrodes which show an absence of  $\text{Mg}^{2+}$  oscillations in a large number of cases, and, in three cases, in association with the presence of  $\text{Ca}^{2+}$  oscillations in the same embryo. An additional indication of the absence of "motion artifacts" is provided by experiments on pHi oscillations in *Xenopus* eggs and embryos (Webb and Nuccitelli, 1981; Grandin and Charbonneau, 1990). During the course of the same study, pHi oscillations, recorded with resin-filled microelectrodes with the same high impedance as the  $\text{Ca}^{2+}$  microelectrodes employed here, had exactly the same amplitude and the same periodicity in artificially activated (nondividing) eggs, embryos, and cleavage-blocked embryos (Grandin and Charbonneau, 1990). Yet, in these three situations, the number of cells greatly differed and, consequently, contractions or cell surface movements were also totally different. This further suggests that, at least in the case of *Xenopus* embryos, this type of microelectrode is insensitive to local mechanical tensions. Finally, it should be stressed that  $\text{Ca}^{2+}$  microelectrodes are totally insensitive to intracellular pH changes. Indeed, during the physiological cytoplasmic alkalization, which starts 8–10 min after triggering of egg activation and is complete 20–30 min later (Webb and Nuccitelli, 1981; Grandin and Charbonneau, 1989), there is no concomitant change in the  $\text{Ca}^{2+}$  level, the  $\text{Ca}^{2+}$  transient rise taking place 1–2 min after triggering of egg activation, that is before the pHi increase (Busa and Nuccitelli, 1985; this study). Likewise, artificially activated *Xenopus* eggs display intracellular pH oscillations, around 0.05 pH unit amplitude (Grandin and Charbonneau, 1990), but no  $\text{Ca}^{2+}$  oscillations (Fig. 5 a, this study). This demonstrates that  $\text{Ca}^{2+}$  oscillations recorded in *Xenopus* embryos are not an artifact due to the cyclic variations of the intracellular pH level.

Besides the unique contribution of the present study, which is the demonstration of periodic  $\text{Ca}^{2+}$  variations during the cell cycle of *Xenopus*, our results strongly suggest that  $\text{Ca}^{2+}$  oscillations are tightly associated to cell division. Indeed, we could not detect  $\text{Ca}^{2+}$  variations in activated, nondividing eggs, and  $\text{Ca}^{2+}$  oscillations were arrested in cleavage-blocked embryos. This indicates that  $\text{Ca}^{2+}$  oscillations should be, at this point, considered as independent of the basic cell cycle. Indeed, the cell cycle of early embryos is determined, independently of cell division, by the oscillation of the cdc2 mitotic kinase activity, which reflects MPF activity cycling (Arion et al., 1988; Labbé et al., 1989b; Felix et al., 1989). This basic cell cycle does not require nuclear components (Dabauvalle et al., 1988), cytoskeletal structures, or even DNA synthesis (Gerhart et al., 1984; Kimelman et al., 1987). In addition, we have recently demonstrated that pHi cycling in *Xenopus* eggs also represented a component of this basic cell cycle (Grandin and Charbonneau, 1990). It is very tempting to postulate a functional relationship between pHi cycling and  $\text{Ca}^{2+}$  cycling in *Xenopus* embryos. Indeed, one could imagine the presence of a specialized vesicular network around the dividing nuclei, that would cyclically release  $\text{Ca}^{2+}$  ions into the cytoplasm in response to the cyclic variation of the transmembrane pH gradient between these hypothetical vesicles and the surrounding cytoplasm. These cyclic variations in cytosolic  $\text{Ca}^{2+}$ , detected here with the  $\text{Ca}^{2+}$ -selective microelectrodes, might in turn play a role in mitosis (see below). However, it is highly speculative to draw such a scheme,

as long as that specialized vesicular network has not been identified. It is also premature, in the sense that we have not yet precisely determined the relationships between the  $Ca^{2+}$  oscillations and the various stages of mitosis, because of technical problems inherent to the opacity of the *Xenopus* eggs.

The suggestion that  $Ca^{2+}$  oscillations in *Xenopus* embryos may play a regulatory role in mitosis is supported by the early finding that microinjection of Ca-EGTA buffers into *Xenopus* blastomeres slowed or even arrested cleavage by specifically lowering the intracellular concentration of  $Ca^{2+}$  ions (Baker and Warner, 1972). An excellent and recent review by Hepler (1989) summarizes all the data on  $Ca^{2+}$  variations during mitosis accumulated to date from various systems, from plant to mammalian cells. First, our basal  $Ca^{2+}$  levels in *Xenopus* embryos, around 0.40  $\mu$ M, are exactly the same as those previously measured in the same system (Busa and Nuccitelli, 1985) and agrees well with basal  $Ca^{2+}$  levels in other systems (see Hepler, 1989). The amplitude of the  $Ca^{2+}$  oscillations in *Xenopus* embryos (mean value: 70 nM) is also comparable to that of  $Ca^{2+}$  transients in other systems, although values sometimes greatly differ within the same system according to different authors (reviewed by Hepler, 1989). A basic difference between our results and those from most other systems is that, in these latter,  $Ca^{2+}$  variations during mitosis consist of transients, while in *Xenopus* embryos, they appear as cyclic oscillations. Thus,  $Ca^{2+}$  transients have been recorded at specific stages of mitosis in sea urchin embryos (Poenie et al., 1985), rat kangaroo PtK1 and PtK2 cells (Keith et al., 1985; Ratan et al., 1986; Poenie et al., 1986), Swiss 3T3 cells (Tombs and Borisy, 1989; Kao et al., 1990), and plant cells (Hepler and Callahan, 1987; Zhang et al., 1990). However, it should be noted that in the absence of serum in the culture medium, Swiss 3T3 cells did not display the previously recorded  $Ca^{2+}$  transients, but rather showed a gradual increase in  $Ca^{2+}$  beginning in late metaphase and extending through anaphase and telophase (Tombs and Borisy, 1989). A similar situation, a gradual increase in  $Ca^{2+}$ , was found in PtK2 cells (Ratan et al., 1988) under conditions apparently similar to those in which  $Ca^{2+}$  transients had been previously recorded. In *Xenopus* embryos, we observe  $Ca^{2+}$  oscillations rather than  $Ca^{2+}$  transients, a situation nevertheless similar to that in medaka eggs (Yoshimoto et al., 1985).

The situation in *Xenopus laevis* embryos, revealed in the present study, appears quite original, because  $Ca^{2+}$  oscillations, taking place with a periodicity of 30 min, seem to be related to the cell division cycle. In addition to the few systems so far utilized to study the role of  $Ca^{2+}$  variations in mitosis (reviewed by Hepler, 1989), *Xenopus* embryos might provide valuable additional information. This is particularly true considering the fact that the embryonic cell cycle of *Xenopus* is one of the most important systems for studying the molecular biology of the so-called cell cycle control proteins, such as cyclins and the cdc2 mitotic kinase.

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