Cycling of Intracellular pH during Cell Division of *Xenopus* Embryos Is a Cytoplasmic Activity Depending on Protein Synthesis and Phosphorylation

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Abstract. In *Xenopus* embryos, the successive and rapid cell divisions that follow fertilization are accompanied by periodic oscillations of intracellular pH (pH_i). Cycling of pH_i occurs in phase with several other oscillatory activities, namely nuclear divisions, M phase-promoting factor (MPF) activity, and surface contraction waves (SCWs). We report that treatments that abolish cycling of MPF activity and the SCWs also suppress the pH_i oscillations, whereas those that block cell division without affecting neither MPF activity nor the SCWs do not suppress the pH_i oscillations. Experiments on enucleated oocytes, matured in vitro and activated, demonstrated that the activity governing the rhythmicity of the pH_i oscillations resided in the cytoplasm of the oocyte. In this respect, the activity responsible for the pH_i oscillations was different from that which drives the SCWs, which necessitated the presence of the oocyte germinal vesicle (Ohsumi et al., 1986), but more closely resembled MPF activity that did not require the presence of the oocyte germinal vesicle (Dabauvalle et al., 1988). In mature eggs enucleated at the time of egg activation, the pH_i oscillations were similar to those in control nucleated eggs, whereas the period between two peaks of SCWs was 35–60 min vs. 20–35 min in nucleated control eggs. Previous studies had shown that the periodicity of SCWs was larger in anucleate egg fragments than in their nucleate counterparts (Sakai and Kubota, 1981), the difference being on the order of 6–15 min (Shinagawa, 1983). However, in these previous studies, enucleation was performed 30–50 min after fertilization. Our results clearly demonstrate that the periodicity of the SCWs is lengthened when the interval between egg activation and enucleation is shortened, thereby providing an easier way to assess the nuclear dependency of the SCWs. Finally, the various possibilities concerning the role of pH_i cycling during cell division are discussed.

In many cells, biological activities have been found to oscillate in relation with a particular physiological state. Oscillatory activities can involve simple ions (oscillations of the intracellular pH or intracellular free calcium activity), molecules (oscillations in the M phase-promoting factor [MPF][1] activity or enzymatic activities), or even more complex assemblies of several reactions, as is the case for the periodic surface contraction waves in dividing amphibian eggs or the periodic rounding-up occurring at each cell cycle in dividing mammalian cells in culture. Oscillatory activities are most easily observed in dividing cells. In such cells, several biological activities have been shown to oscillate in phase with each cell division.

The egg of *Xenopus laevis* is probably the only cell in which several oscillatory activities can be recorded. So far, three main types of oscillatory activities have been shown to operate in *Xenopus* eggs during the embryonic development: surface contraction waves, MPF activity, and oscillations of the intracellular pH level. Surface contraction waves (SCWs), first observed by Hara (1971), occur periodically during *Xenopus* embryogenesis, with the same periodicity as the cleavage cycles. These SCWs appear to be independent of nuclear activities as they also take place in anucleate egg fragments (Sawai, 1979; Hara et al., 1980), although at a lower periodicity than in nucleate egg fragments (Sakai and Kubota, 1981; Shinagawa, 1983). Moreover, oocytes induced to mature after removal of the germinal vesicle (nucleus) initially showed no SCWs when activated (Ohsumi et al., 1986). MPF, a universal M phase-promoting factor (Masui and Markert, 1971), has also been found to oscillate in *Xenopus* embryos (Gerhart et al., 1984). Contrary to the SCWs, MPF activity rises during oocyte maturation even in the absence of the germinal vesicle (Masui, 1972) and oscillates in activated enucleated oocytes (Dabauvalle et al., 1988). Oscillations of the intracellular pH (pH_i) level have been described by Lee and Steinhardt (1981) and Webb and Nuccitelli (1981). Interestingly, Lee and Steinhardt (1981)

1. *Abbreviations used in this paper*: MPF, M phase-promoting factor; SCW, surface contraction waves; 6-DMAP, 6-dimethylaminopurine.

considered these pH_i oscillations, that they could record only in a minority of cases, as artefacts. However, further experiments provided clear evidence against Lee and Steinhardt's arguments (1981) and demonstrated that pH_i oscillations were not recording artefacts (Webb and Nuccitelli, 1982). The pH_i oscillations were found to have a periodicity equal to that of the cell cycle, but out of phase with it, as far as cleavage (cytodieresis) was considered (Webb and Nuccitelli, 1981).

In this study, we have investigated the relationships between the three types of oscillations that take place during *Xenopus* embryo cell division, namely pH_i oscillations, surface contraction waves, and oscillations in MPF activity. We report that the activity at the origin of the pHi oscillations resides in the cytoplasm, unlike the surface contraction waves, but similarly to the cycling activity of MPF.

Materials and Methods

Obtaining eggs and sperm from mature *Xenopus laevis* reared in the laboratory, as well as activating and fertilizing eggs were performed according to previously described procedures (Charbonneau et al., 1986). The physiological solution, Fl, modified from Hollinger and Corton (1980), contained (in millimolar): 31.2, NaCl; 1.8, KCl; 1.0, $CaCl_2$; 0.1, $MgCl_2$; 1.9, NaOH; and 2.0, $NaHCO_3$; buffered with 10 mM Hepes (pH 7.4). Mature jellied eggs or embryos were dejellied by gentle swirling (4–8 min) in Fl containing 2% cysteine (pH 7.8). For pH microelectrode impalement (see below), an Fl solution buffered with 10 mM Capso (pH 8.5–9.0) was used to visualize on the pen recorder the deflexion of the pH trace, indicating the entry of the microelectrode into the egg cytoplasm.

Various chemicals and drugs were used to block cleavage at particular levels of the cell cycle. Nocodazole, an inhibitor of microtubule assembly, was prepared as a stock solution of 1 mg/ml in 50% H_2O–50% DMSO and used at a final concentration of 10 μg/ml. Cytochalasin B, an inhibitor of microfilament polymerization, was prepared as a stock solution of 5 mg/ml in DMSO, and used at a final concentration of 5 μg/ml. Cycloheximide, an inhibitor of protein synthesis, was used at 100–200 μg/ml (stock solution: 5 mg/ml in H_2O). Aphidicolin, an inhibitor of DNA polymerase and hence of DNA replication, was dissolved in 50% ethanol–50% 1,2-propane diol (1 mg/ml) and injected (40–50 nl) into embryos, 30–45 min after fertilization. Control embryos were injected with the mixture 1,2-propane diol-ethanol. 6-DMAP (6-dimethylaminopurine), which blocks the cell cycle without affecting protein synthesis (Rebhun et al., 1973) by inhibiting protein kinase activity and triggering a dramatic global dephosphorylation (Néant and Guerrier, 1988), was used at 300 to 600 μM (stock solution: 15 mM in H_2O).

Intracellular pH (pH_i) was measured using microelectrodes containing at their tips an H^+-selective neutral carrier-based resin (Amman et al., 1981), purchased from Fluka Chemical Corp. (Buchs, Switzerland) and fabricated and calibrated as described previously (Charbonneau et al., 1985; Grandin and Charbonneau, 1989*a*). The pH response of these microelectrodes was 54–61 mV per pH unit, with a full response time of a few seconds. Membrane potential recorded simultaneously in the same egg with a voltage microelectrode (GC 150F; Clark Electromedical Instruments, Reading, England), filled with 3 M KCl, 10 mM EDTA and 10 mM potassium citrate, was subtracted from the pH microelectrode output at the pen recorder (Linseis) input, to give a trace corresponding to pH_i. Electrical recordings were performed in 4-ml tissue culture plastic dishes (60 × 15 mm), with a center well (Falcon Labware, Oxnard, CA). It is important to note that for each experimental condition tested, control experiments were run at the same time on a second electrophysiological set-up placed exactly under the same conditions. Microinjections were performed as described in Grandin and Charbonneau (1989*b*).

Enucleation procedures were applied on immature oocytes or mature eggs. Full-grown oocytes (stage VI in Dumont, 1972) were manually defolliculated in OR_2, modified from Wallace et al. (1973), which contained (in millimolar): 82.5, NaCl; 2.5, KCl; 1.0, $CaCl_2$; 1.0, $MgCl_2$; 3.8, NaOH; 2.0, $NaHCO_3$, buffered with 10 mM Hepes and adjusted at pH 7.4. The oocytes were enucleated according to a method similar to that described by Ford and Gurdon (1977) and shown in Fig. 1. The healing solution, in which the oocytes recovered for ~1.5 h after enucleation, contained 90 mM KH_2PO_4-K_2HPO_4 (pH 7.2), 10 mM NaCl, and 1 mM $MgSO_4$. Mature dejellied eggs (metaphase II stage of meiosis) were enucleated according to the same method as for oocytes, but were allowed to heal either in Fl solution (measurements of pH_i or SCWs) or in the healing solution (measurements of MPF activity). In the latter case, MPF activity of control nucleated eggs was also measured in the healing solution. Eggs were activated with the syringe needle used for enucleation.

To assay MPF activity, one or two eggs were placed on a piece of parafilm. Excess solution around the eggs was carefully removed and the eggs immediately homogenized in extraction buffer (80 mM sodium β-glycerophosphate, 15 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, pH 7.4, 1 μl per egg), by several passages through an Eppendorf pipette tip. The crude extracts were diluted twofold in the extraction buffer, sedimented at 3,500 *g*, and ~40 nl of the soluble fraction were immediately microinjected into stage VI immature *Xenopus* oocytes (four for each point). Successful oocyte maturation was scored by visualizing under a stereomicroscope the germinal vesicle moving up to the animal pole and its subsequent breakdown (GVBD), ~1.5–2.5 h after microinjection, ending in the formation of the maturation spot with the extremity of the second metaphase furrow in its center. In some cases, successful maturation was confirmed by dissecting on glutaraldehyde-fixed oocytes (2–3 h in 2.5% glutaraldehyde in Fl) the piece of cortex corresponding to the animal pole and staining, after squashing between a glass slide and a coverslip, the chromosomes with bisbenzimide as described in Grandin and Charbonneau (1989*b*).

For recording of the surface contraction waves (SCWs), dejellied nucleated eggs were activated by pricking, whereas dejellied enucleated eggs were activated by the syringe needle used to remove the nucleus. At the time the animal cap completed the relaxation following the cortical contraction, ~20 min after activation, the nucleated eggs were treated with 0.5% protease (from *Streptomyces griseus*, type XIV from Sigma Chemical Co., St. Louis, MO) for 5–10 min to remove the vitelline envelope (Ohsumi et al., 1986). Enucleated eggs were dechorionated twenty min after enucleation, by treating for 1–3 min with a cocktail containing 1.25 mg/ml papain, 6.2 mM cysteine, pH 2.8, in Fl solution, as described by Richter (1980). The reaction was stopped with 3 mM monoiodo acetic acid (in H_2O, 10 min). In these experiments, the control nucleated eggs were similarly dechorionated. After rinsing following protease or cystein–papain treatment, dechorionated eggs were transferred to various agarose-coated control and experimental dishes. The diameters of the dechorionated eggs, placed animal pole up, were measured from above, under a stereomicroscope, every 5 min (Shinagawa, 1983). Control and experimental eggs were placed in exactly similar conditions of temperature.

To visualize the state of the nucleus and chromosomes with respect to pH_i oscillations, eggs that were impaled with potential and pH microelectrodes were removed from the recording chamber at intervals and immediately fixed for 24 h in Smith's fixative (Humason, 1972). After dehydration in series of ethanol and butylic alcohol, and embedding in paraffin, eggs were sectioned at 5 μm and stained with bisbenzimide to detect chromatin and chromosomes.

Results

Relationships between Intracellular pH Oscillations and Mitosis

As first shown by Webb and Nuccitelli (1981), *Xenopus* egg fertilization is accompanied by a permanent increase in intracellular pH (pH_i) which is followed during early embryogenesis by periodic oscillations of pH_i around its elevated value, each oscillation corresponding to one cell division (Fig. 2). In this study, the exact relationship between the cell cycle and pH_i oscillations was analyzed by fixing eggs impaled with pH microelectrodes at various times during the oscillation cycle and examining sections through the nuclei with the light microscope (Fig. 2). This clearly showed that the alkaline peak of the pH_i oscillation corresponded to mitosis, more precisely the metaphase stage, which also corresponded to the completion of cleavage (cytodieresis), whereas at the acidic peak of the oscillation, nuclei were interphasic (Fig. 2). A point that had been previously verified (Webb and Nuccitelli, 1982) is that in artificially activated eggs, pH_i oscillations were exactly similar to those in em-

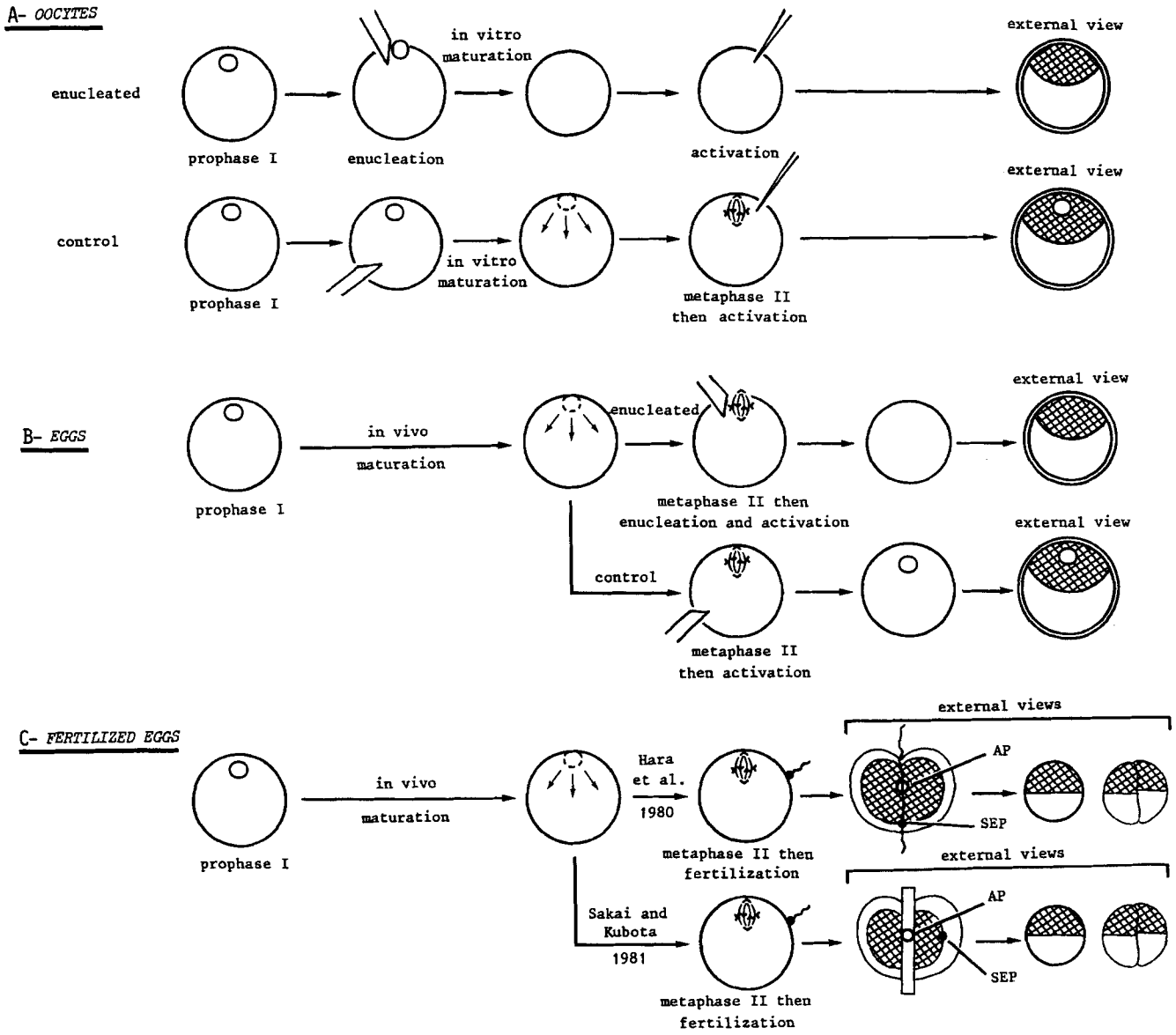

Figure 1. Schematic representation of the methods used for enucleating oocytes and eggs of *Xenopus laevis*. (*A*) In oocytes, the germinal vesicle was removed before maturation. Immature full-grown oocytes (prophase I-arrested) were defolliculated with forceps. A small incision was made at the animal pole with a syringe needle (microlance 25 G 5/8, 0.5 × 16 mm). The syringe needle was then removed carefully, and the oocyte was slightly squeezed with forceps until the germinal vesicle appeared in the slit and emerged freely in the external solution, OR_2 (see Ford and Gurdon, 1977). Control (nucleated) oocytes were also punctured with a syringe needle at the equator or in the vegetal hemisphere. Oocytes were subsequently allowed to recover for 1.5 h in the healing solution and transferred back to OR_2 solution for a few hours. Oocytes were then induced to mature after addition of progesterone (3 μM). In oocytes stimulated in vitro with progesterone or in vivo (see below, *B* and *C*), maturation was characterized by a moving up of the germinal vesicle to the animal pole and disruption of the germinal vesicle envelope, allowing a mixing between nuclear and cytoplasmic materials (represented by the arrows in the schemes). In the absence of the germinal vesicle, successful maturation in enucleated oocytes was assessed by visualizing after egg activation (induced by pricking the egg cortex with a 50-μm-diam glass micropipette) the cortical contraction and the elevation of the vitelline envelope (a consequence of cortical granule exocytosis), two early events of egg activation. (*B*) In eggs, the nucleus was removed at the metaphase II stage of meiosis. Eggs matured in vivo were dejellied with cysteine, enucleated with a syringe needle, and allowed to recover in FI solution (measurements of pH_i or SCWs) or in the healing solution (measurements of MPF activity). Control (nucleated) eggs were punctured with a syringe needle at any place distinct from the animal pole. In both enucleated and nucleated eggs, activation was triggered by the syringe needle. (*C*) Comparison with the methods used by Hara et al. (1980) and Sakai and Kubota (1981). Separation of fertilized eggs of *Xenopus* into a nucleate and an anucleate fragment was realized either with a newborn human hair, 40–50 min after fertilization (Hara et al., 1980) or with a glass rod, 30–45 min after fertilization (Sakai and Kubota, 1981). The dividing fragment is regarded as the nucleated one. *AP*, animal pole; *SEP*, sperm entry point.

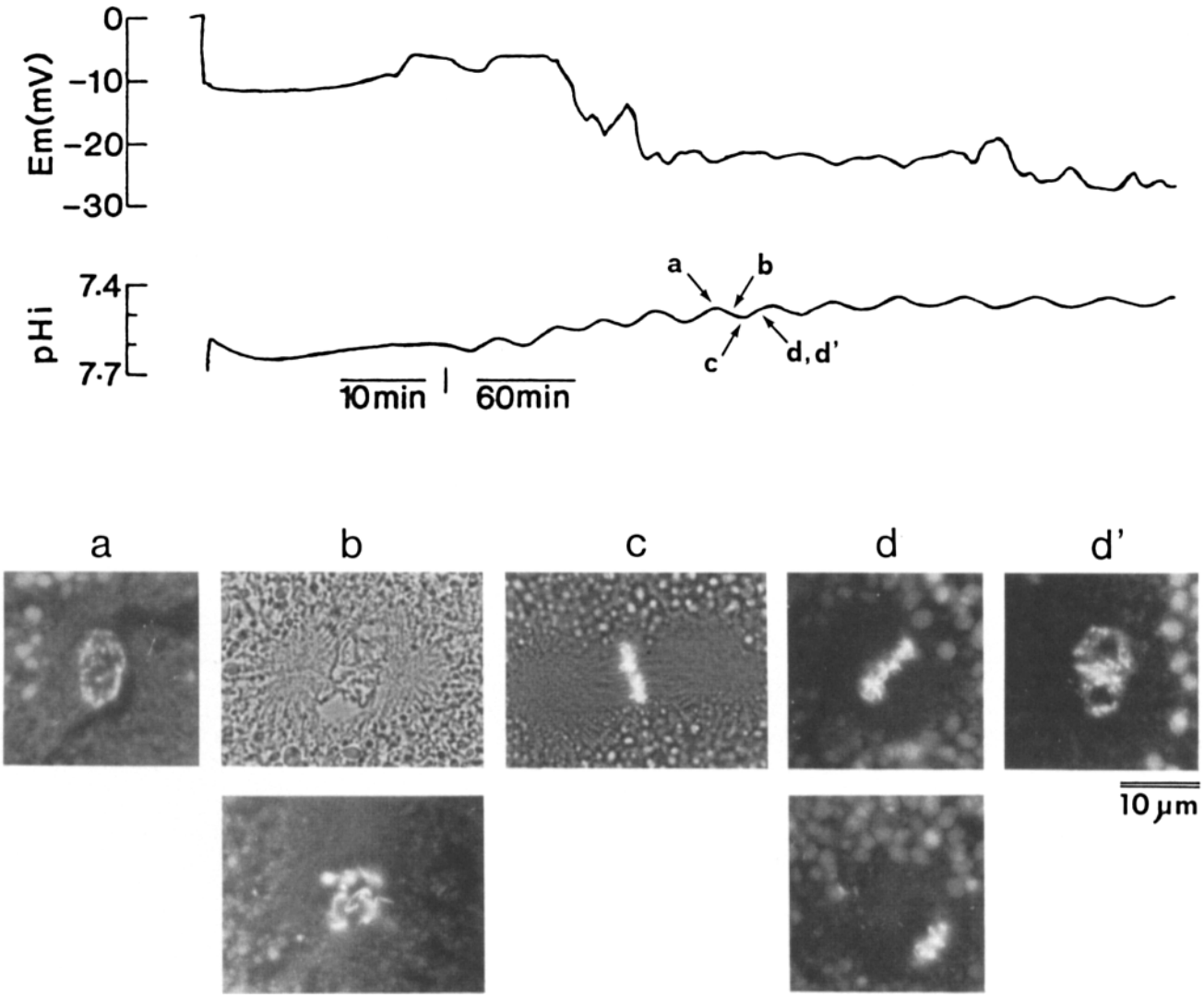

Figure 2. Intracellular pH variations during the early development of *Xenopus laevis* embryos. This egg was impaled with a potential microelectrode (*top trace*) and a pH microelectrode (*bottom trace*), 30 min after fertilization. At this time, pH_i has already attained its plateau value, ~pH 7.7, characteristic of activated or fertilized eggs. Starting ~60 min after fertilization, pH_i oscillated in phase with the cell cycle. The alkaline peak of the first pH_i oscillation corresponded to the first cleavage (1 h 20 min after fertilization at 23–24°C). Each alkaline peak of the successive pH_i oscillations indicated the successive cell divisions of the embryo (cytodieresis). At the same time, the nuclei were metaphasic (metaphase of the next division), as seen on sections stained with bisbenzimide (*c*). Each acidic peak of the pH_i oscillations corresponded to interphasic nuclei (*a*). In embryos fixed between the acidic peak and the alkaline peak, the nuclei were at the prophase stage of mitosis (*top panel* in *b*: the two asters surround the prophasic nucleus, as seen with the light microscope; *bottom panel* in *b*: prophasic chromosomes stained with bisbenzimide), whereas in embryos fixed between the alkaline peak and the acidic peak, the nuclei were at the anaphase (*top panel* in *d*: one set of anaphasic chromosomes; *bottom panel* in *d*: the other set of anaphasic chromosomes in the same nucleus) or telophase (photograph *d'*) stages of mitosis. The period of pH_i cycling, ~30 min, was seen to correlate with the cell division cycle, and was lengthened as the latter was slowed down, at a lower temperature, for instance.

bryos. The pH_i oscillation represents therefore an autonomous oscillator that does not require sperm material or cell division.

Treatments that Affect the Cycling of MPF Activity and Surface Contraction Waves also Affect pH_i Oscillations

To characterize the origin of the pH_i oscillations, pH_i was measured in eggs treated with drugs known to affect two other main oscillatory activities, MPF activity, and surface contraction waves (SCWs).

Cycloheximide, which inhibits protein synthesis, was found to completely abolish the pH_i oscillations in fertilized or artificially activated eggs (Fig. 3). Cycloheximide is known to inhibit the cycling of MPF activity (Gerhart et al., 1984) and to suppress the SCWs (Kimelman et al., 1987), as confirmed in this study (Fig. 3). Together with protein syntheses, protein phosphorylations are known to be involved in egg activation and the subsequent cell divisions. 6-DMAP (6-dimethylaminopurine) is an inhibitor of protein phosphorylation which does not interfere with protein synthesis (Rebhun et al., 1974) and operates in sea urchin and

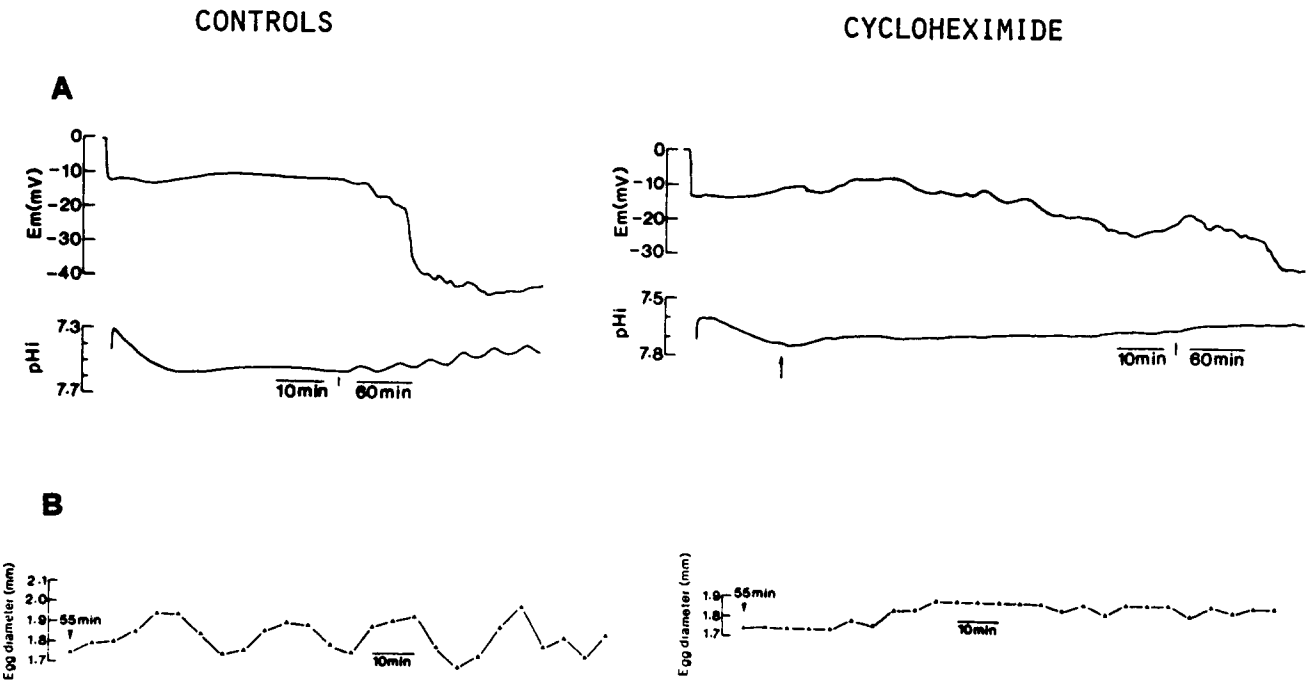

Figure 3. Effects of inhibition of protein synthesis by cycloheximide (100 μg/ml) on pH_i oscillations of *Xenopus* embryos (*A*) and SCWs of artificially activated eggs of *Xenopus* (*B*). (*A*) pH_i recordings and membrane potential recordings (*Em*) in control embryos (*left*) and cycloheximide-treated embryos (*right*). As mentioned in Fig. 2, E_m and pH_i were recorded simultaneously in the same embryo with two intracellular microelectrodes. Membrane potential (E_m), recorded by the potential microelectrode, was subtracted at the pen recorder input, from the total signal recorded by the pH microelectrode, which corresponded to the voltage proportional to the H^+ ion activity measured plus the membrane potential (pH_i plus Em). pH_i oscillations were blocked by cycloheximide. In these experiments, both control and cycloheximide-treated embryos were impaled with microelectrodes 8 min after insemination. Note the fertilization-associated pH_i increase starting ~10 min after insemination, that is ~5 min after fertilization. Cycloheximide was added 25 min after insemination (*arrow*); cell division was blocked as early as the first cleavage. A complete disappearance of pH_i cycling after cycloheximide treatment was observed in all six experiments performed. (*B*) SCWs measurements, which were started 55 min after egg activation (*arrowheads*); cycloheximide treatment was applied 50 min after egg activation. In all cases (nine experiments, see Table I), the blockade of protein synthesis resulted in a complete disappearance of SCWs.

starfish eggs (Néant and Guerrier, 1988) and in *Xenopus* egg cytoplasmic extracts (Felix et al., 1989). In eggs preincubated in a solution containing 6-DMAP and subsequently activated, the SCWs and pH_i oscillations were strongly perturbed (Fig. 4). The periodicity of the SCWs was increased with respect to control eggs, and accompanied by a progressive and large increase in egg diameter (Fig. 4 and Table I). In some cases, the SCWs completely ceased (Fig. 4). Similarly, pH_i oscillations were considerably lengthened and eventually disappeared, accompanied in some cases by a progressive alkalinization of the cytoplasm (Fig. 4).

Treatments that Block Cleavage without Affecting the Cycling of MPF Activity or SCWs Do Not Affect pH_i Oscillations

Nocodazole, an inhibitor of microtubule assembly, blocks cleavage by preventing nuclear divisions, but has no effect on MPF activity cycling (Gerhart et al., 1984) and SCWs (Kimelman et al., 1987), although Shinagawa (1983) demonstrated that another inhibitor of microtubule assembly, colchicine, produced a lengthening of the periodicity of SCWs. In this study, fertilized eggs treated with nocodazole failed to cleave, but nevertheless displayed pH_i oscillations with the same periodicity as in dividing embryos (Fig. 5). Similarly, when cell division was blocked by microinjection of aphidicolin, an inhibitor of DNA synthesis, pH_i oscillations were not inhibited (Fig. 5). MPF activity cycling and SCWs have also been shown to be independent of DNA synthesis (Kimelman et al., 1987). Finally, blocking cytodieresis, but not cytokinesis, with cytochalasin B, an inhibitor of microfilament assembly, did not result in an inhibition of pH_i oscillations that remained similar to those in untreated embryos (Fig. 5).

Nuclear Material Is Not Needed for Intracellular pH Cycling

Nuclear material contained in the oocyte germinal vesicle is released into the cytoplasm after germinal vesicle breakdown occurring during oocyte maturation. Material contained in this nuclear sap has been shown to participate in the subsequent SCWs (Ohsumi et al., 1986; Dabauvalle et al., 1988), but not in MPF activity cycling (Dabauvalle et al., 1988), which occurs after activation of the mature oocyte. Enucleation therefore represents an appropriate procedure to determine whether the pH_i oscillations have the same origin as the SCWs, or are independent of nuclear activity as is the case for MPF activity. In enucleated oocytes, subsequently matured with progesterone, pH_i oscillations were still generated (Fig. 6 *A*). To confirm the absence of a role of the nucleus in pH_i cycling, eggs were also enucle-

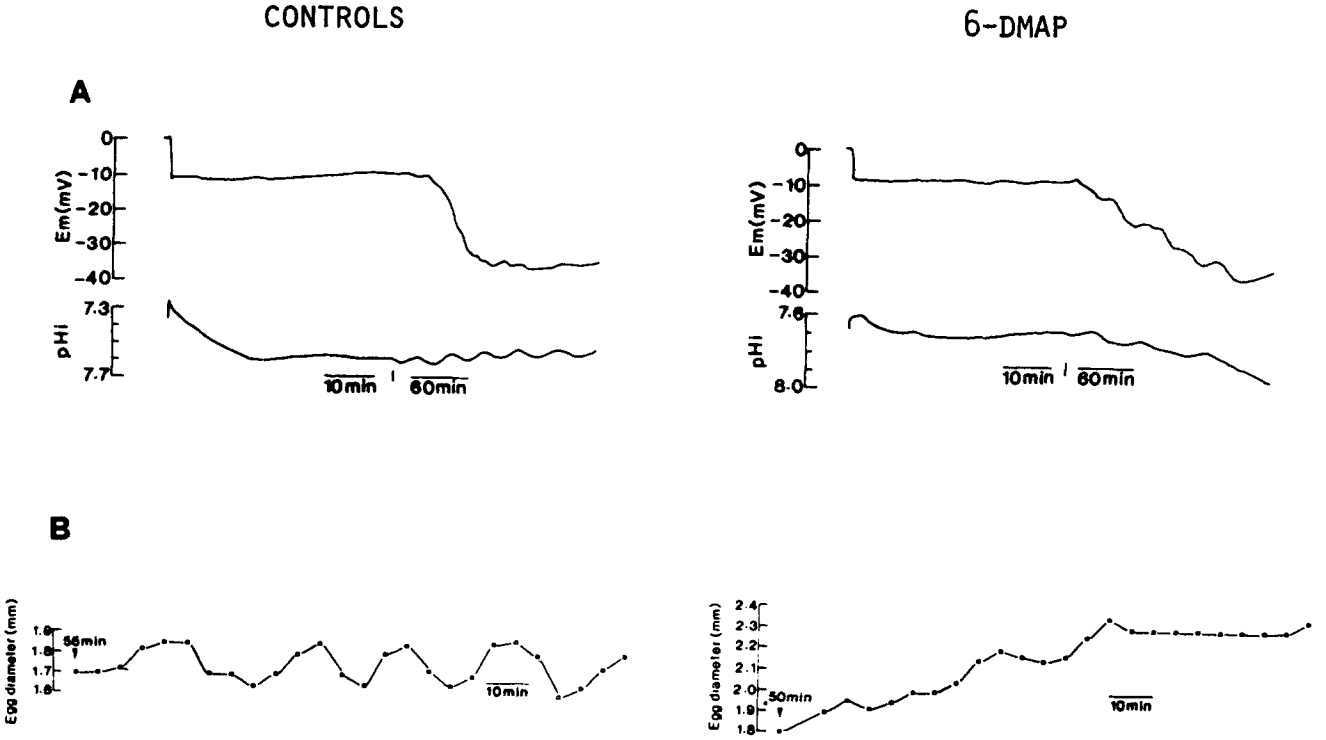

Figure 4. Effects of protein phosphorylation inhibition by 300 μM 6-DMAP on pH_i oscillations of *Xenopus* embryos (*A*) and SCWs of artificially activated *Xenopus* eggs (*B*). 6-DMAP, which in this experiment was applied at the time the egg was impaled with the microelectrodes (15 min after insemination), resulted in a lengthening of pH_i oscillations with respect to the control (impaled 10 min after insemination). The oscillations eventually disappeared, concomitantly with a sustained alkalinization of the cytoplasm. Similar patterns were observed in all 10 experiments performed. The SCWs shown here were measured starting 55 min (control) and 50 min (6-DMAP, time of treatment: 45 min) after egg activation. The period of SCW cycling in 6-DMAP-treated eggs was slightly lengthened with respect to control eggs (see also Table I). In some cases, as here, the SCWs of 6-DMAP-treated eggs ceased. In all cases, the treatment was accompanied by an increase in egg diameter (see Table I). These effects of 6-DMAP on SCWs were observed in 18 experiments.

Table 1. Effects of 6-DMAP, Cycloheximide,‡ and Nuclear Material on the SCWs of Activated Eggs of Xenopus laevis§*

Treatment	Egg diameter before the SCWs‖	Increase in egg diameter during the relaxation phase¶	Decrease in egg diameter during the contraction phase	Periodicity of the SCWs**	Egg diameter (off-peak) at the end of treatment‡‡
		mm		*min*	*mm*
Controls	1.64 ± 0.20§§ (9 eggs)	0.20 ± 0.08 (35 SCW, 10 eggs)	0.19 ± 0.07 (33 SCW, 10 eggs)	25.70 ± 6.00 (33 SCW, 10 eggs)	1.67 ± 0.08 (9 eggs)
6-DMAP	1.65 ± 0.15 (18 eggs)	0.23 ± 0.06 (44 SCW, 18 eggs)	0.11 ± 0.06 (41 SCW, 18 eggs)	28.70 ± 6.90 (39 SCW, 18 eggs)	2.00 ± 0.22 (17 eggs)
Cycloheximide	1.63 ± 0.09 (9 eggs)	No SCW	No SCW	No SCW	1.70 ± 0.14 (9 eggs)
Nucleated (controls)	1.73 ± 0.08 (9 eggs)	0.23 ± 0.08 (44 SCW, 12 eggs)	0.24 ± 0.08 (37 SCW, 12 eggs)	25.73 ± 5.44 (34 SCW, 12 eggs)	1.75 ± 0.06 (12 eggs)
Enucleated‖‖	1.58 ± 0.12 (7 eggs)	0.29 ± 0.07 (15 SCW, 7 eggs)	0.26 ± 0.08 (12 SCW, 7 eggs)	44.00 ± 8.20 (13 SCW, 7 eggs)	1.58 ± 0.16 (3 eggs)
	1.50 ± 0.00 (4 eggs)	No SCW	No SCW	No SCW	1.83 ± 0.04 (4 eggs)

* 6-DMAP was used at 300 μM (final concentration) to inhibit protein phosphorylation.
‡ Cycloheximide (final concentration: 100 μg/ml) was used to inhibit protein synthesis.
§ Unactivated dejellied eggs were activated by pricking with a glass micropipette (5–10 μm tip diameter) or with the syringe needle (0.5-mm diameter) used for enucleation, and, in all cases, SCWs were recorded in F1 solution after removal of the vitelline envelope either with protease (6-DMAP- and cycloheximide-treated eggs and their controls) or with cystein-papain (enucleated eggs and their controls) as described in Materials and Methods.
‖ This value was obtained 40–50 min after egg activation; the first SCW began 50–60 min after egg activation. In control eggs, this value also corresponds to the minimal egg diameter at the peak of the contraction phase of each SCW (see Figs. 2 and 7).
¶ The so-called "relaxation phase" refers to the "flattening" of the egg after each SCW, a term used by other authors.
** Periodicity corresponds to the duration of each SCW, measured between two successive peaks of contraction.
‡‡ This value was obtained 2–3 h after egg activation. In eggs which still displayed SCWs at the end of the treatment, the diameter given here corresponds to the minimal diameter during a contraction phase (off-peak value of the trace; see Figs. 2 and 7).
§§ All values are mean values ± SD.
‖‖ Two classes of enucleated eggs were clearly discernible. A first class of eggs still displayed SCWs after treatment, whereas the second class corresponds to eggs in which SCWs have totally disappeared.

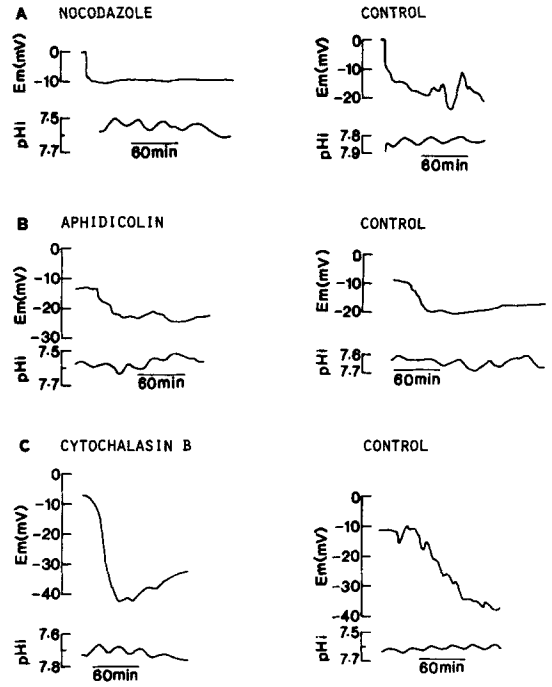

Figure 5. Intracellular pH cycling in cleavage-blocked *Xenopus* embryos, using nocodazole (*A*), aphidicolin (*B*), and cytochalasin B (*C*). All treatments were found to block cell division, either as a result from the inhibition of microtubule assembly (nocodazole), or microfilament assembly (cytochalasin B) or DNA synthesis (aphidicolin). In such arrested embryos, pH_i was nevertheless found to oscillate as in their respective controls. (*A*) 10 μg/ml nocodazole was applied around the embryos 1 h 30 min after insemination, and pH_i recording started at the same time. The control embryo presented here was impaled with microelectrodes 2 h after insemination. These traces are representative of four experiments (four treated plus four controls). (*B*) Aphidicolin was injected into embryos (40 nl of a 1 mg/ml stock solution) 20 min after insemination; pH_i recording began 1 h 55 min after insemination. Controls were injected with 40 nl of 1,2-propane diol-ethanol (0.5–0.5) 20 min after insemination; this trace starts 1 h 30 min after insemination. Results were similar in all four experiments performed (four treated plus four controls). (*C*) 5 μg/ml cytochalasin B was applied 30 min after insemination. In this experiment, both the control and the treated embryos were impaled with microelectrodes 1 h after insemination. In all four experiments performed (four treated plus four controls), an absence of effect of cytochalasin B on pH_i oscillations was observed.

ated at the end of oocyte maturation. At this stage, the mature egg laid by the female is at the metaphase II stage of meiosis, the metaphasic chromosomes being located at the animal pole, just beneath the plasma membrane. In such enucleated eggs, pH_i oscillations were found to be exactly similar to those taking place in control nucleated eggs (Fig. 6 *B*).

Nuclear Material Regulates the Periodicity of Surface Contraction Waves

In a previous study, it had been demonstrated that anucleate egg fragments mechanically separated from the embryos 30–50 min after fertilization had a longer periodicity in their SCWs than their nucleate counterparts (Shinagawa, 1983). However, the difference between anucleate and nucleate egg fragments was small, on the order of a few minutes (Shinagawa, 1983), which explained that earlier investigators had missed this phenomenon (Hara et al., 1980). We reasoned that this interval might be increased if the delay between egg activation and enucleation were shortened. This can be achieved by enucleating eggs at the time of activation. In addition, measurements of MPF activity in enucleated eggs had not been previously reported in the literature. It was therefore necessary to obtain a pattern for these two oscillatory activities that could be compared with that of pH_i oscillations in enucleated eggs. MPF activity was found to cycle in such enucleated eggs with a periodicity similar to that in nucleated eggs (Fig. 7), a finding that was not too surprising since MPF activity had been found to cycle after activation in matured enucleated oocytes (Dabauvalle et al., 1988). Fig. 8 shows the results of measurements of SCWs in enucleated and control eggs. The periodicity of the waves

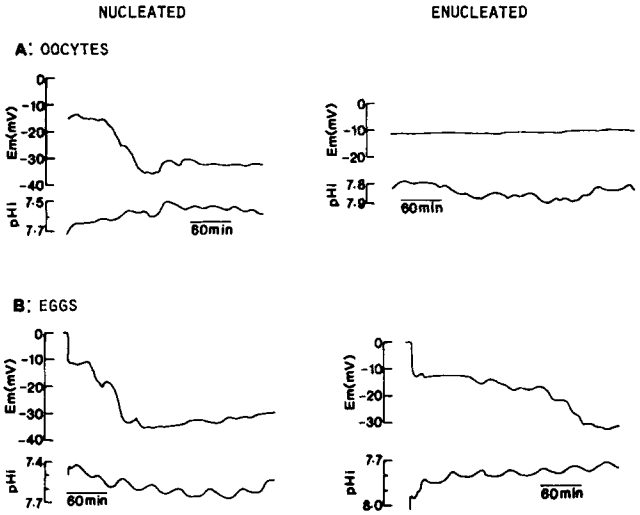

Figure 6. Absence of a role of nuclear material in the generation of pH_i oscillations. (*A*) Oocytes were enucleated (immature stage VI), allowed to heal, matured with 3 μM progesterone, and activated by pricking. In the experiments shown here, the control (also incubated in the healing solution and matured with progesterone) and the enucleated oocytes were respectively impaled with microelectrodes (in F1 solution) 50 min and 2 h 20 min after egg activation, which in both cases was triggered 10 h after progesterone addition, ~2 h after completion of oocyte maturation. In enucleated oocytes, successful oocyte maturation and egg activation were assessed, in the absence of the nucleus, by observing the cortical contraction and vitelline envelope lifting up. Intracellular pH oscillations were present in enucleated oocytes, but were more or less regular. However, this was also the case for control oocytes. In addition, the occasional observation, in both enucleated and nucleated oocytes, of a delay of ~2 h between egg activation and the beginning of pH_i cycling, instead of 1 h in in vivo matured eggs, seemed to be due to the procedure used for oocyte maturation rather than to the absence of the nucleus. Oscillations of pH_i were observed in 11 enucleated oocytes and 11 controls. (*B*) Eggs (matured in vivo and dejellied) were enucleated with a syringe needle, which also triggered egg activation, and allowed to heal in F1 solution. Control eggs were activated by pricking at the same time. The two traces presented here start 1 h after egg activation. Intracellular pH oscillations were exactly similar in enucleated and nucleated eggs, as was verified in six experiments (six enucleated plus eight nucleated).

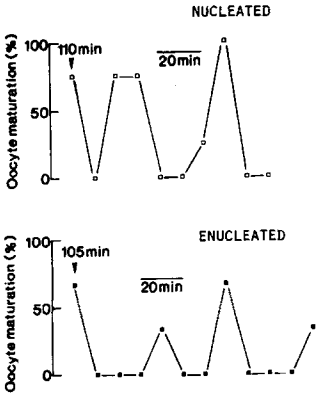

Figure 7. MPF cycling in enucleated eggs. Mature dejellied eggs were activated upon enucleation with a syringe needle or with a glass micropipette (controls), and MPF activity measured at intervals after activation as explained in Materials and Methods. For both enucleated and control eggs, MPF activity was measured in the healing solution. The arrowheads (110 and 105 min) indicate the times after egg activation at which measurements were started. In both enucleated and nucleated eggs, MPF was found to cycle, although not precisely with the same periodicity (two experiments for each).

was found to be much longer in enucleated eggs (35–60 min interval between two waves; mean value 44 min, see Table I) than in control eggs (20–35-min interval between two waves; mean value 25 min, see Table I). In this study, the differences in SCW cycling between these two populations of eggs were much larger than in the experiments in which SCW cycling was compared in nucleate fragments and in nonnucleate fragments isolated 30–50 min after fertilization (Shinagawa, 1983). In addition, in 4 out of 11 experiments, enucleated eggs were completely devoid of SCW (Table I).

Discussion

The role of intracellular pH oscillations during the cell cycle of *Xenopus* embryos, first described by Webb and Nuccitelli (1981; 1982), is totally unknown. On the same material, Lee and Steinhardt (1981) were unable to detect any cycling of pH_i in the majority of embryos. In only two recordings could these authors detect a very small pH cycle that they interpreted as being an artefact due to the fact that the two electrodes were inserted in partially uncoupled blastomeres with slightly different membrane potential cycling, thus generating an artefactual mirror image. However, pH_i cycling remained well visible in uncleaving embryos (this study) or in artificially activated eggs (Webb and Nuccitelli, 1982; this study), as well as in embryos in which cleavage-associated membrane hyperpolarizations had been suppressed (Webb and Nuccitelli, 1982).

In this report, we have tried to further analyze these pH_i oscillations by affecting metabolic events classically known to be key components of the cell cycle, as well as by studying their relationships with two other relatively well-studied oscillatory activities taking place during the same period, namely MPF activity cycling and periodic surface contraction waves.

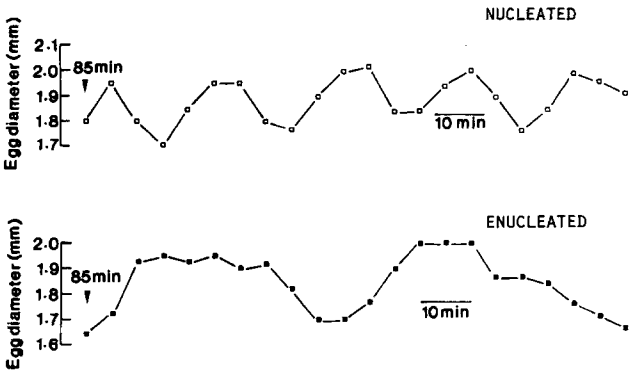

Figure 8. SCW cycling in enucleated eggs. Mature dejellied eggs were activated upon enucleation or by pricking (controls), and their vitelline envelopes removed as described in Materials and Methods. SCWs were measured in F1 solution, 85 min after egg activation in these experiments. This is an example in which enucleated eggs displayed a considerable lengthening of the period between two waves. The mean values and number of experiments are given in Table I. In other cases, the SCWs were no longer generated by the enucleated eggs (see Table I).

Intracellular pH Cycling Depends on Protein Synthesis and Phosphorylation

Intracellular pH oscillations were found to be completely suppressed when protein synthesis was inhibited and considerably lengthened and attenuated when protein phosphorylation was inhibited. Of course, in the two situations, cell division was totally suppressed. Interestingly, MPF activity cycling (Gerhart et al., 1984) and the SCWs (Kimelman et al., 1987) are also inhibited by cycloheximide. In addition, the inhibition of protein phosphorylation by 6-DMAP produced a lengthening and an attenuation of the SCWs in the eggs, which also became abnormally large. 6-DMAP seems to act mainly as a protein kinase inhibitor (Néant and Guerrier, 1988; Felix et al., 1989). However, as an ATP analogue, 6-DMAP could inhibit other processes than protein phosphorylation, which may be responsible for the increase in size of the eggs observed in this study. The histone H1 kinase activity of one of the components of MPF has also been shown to depend on both protein synthesis and phosphorylation (Arion and Meijer, 1989).

The requirement for pH_i oscillations of synthesis and phosphorylation of one or several proteins is not simply due to the resulting arrest of cell division. Indeed, our results clearly show that when cell division was inhibited by other means, such as an inhibition of microfilament or microtubule assembly, or an inhibition of DNA synthesis, pH_i continued to periodically oscillate in phase with neighboring untreated embryos. These latter treatments have been shown to suppress cell division, but not other aspects of the cell cycle that are represented by MPF activity and SCW cycling (Hara et al., 1980; Gerhart et al., 1984; Kimelman et al., 1987). Therefore, our results suggest that pH_i cycling also represents a component of this cell cycle, still operating when cell division is arrested.

To make the distinction between cell division and cell cycle (or basic cycle) clearer, it is important to stress that it is now admitted that 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., 1989; Felix et al., 1989). This basic cycle, which is the "master oscillator" postulated by Hara et al. (1980), is responsible for the rapid alternation between interphase and mitosis, and does not require nuclear components (Dabauvalle et al., 1988) or cytoskeletal structures (Gerhart et al., 1984; Kirschner et al., 1985; Kimelman et al., 1987).

Nuclear Material Is Necessary for the Generation of SCWs But Not for pH_i and MPF Activity Cycling

On first analysis, the relationships between pH_i oscillations and the presence of the oocyte nucleus are similar to those between MPF activity and the presence of the germinal vesicle. Indeed, it was recently demonstrated that oocytes induced to mature after removal of their germinal vesicle did not exhibit any SCWs when they were activated (Ohsumi et al., 1986). In fact, some of these enucleated matured oocytes exhibited delayed transient flattenings of longer periodicity than in nucleated eggs (Ohsumi et al., 1986). Similar observations made by others (Dabauvalle et al., 1988) also suggest that some material removed by enucleation is slowly resynthesized, since normal SCWs become visible after a time delay proportional to the period of time between enucleation and egg activation. On the other hand, the presence of the nucleus is not required for MPF activity cycling, as shown by removal of the nucleus in immature oocytes (Dabauvalle et al., 1988) or in mature eggs (present results). Similarly, the presence of the nucleus was not required for pH_i cycling, either in oocytes or in eggs, thereby demonstrating that pH_i oscillations represent a cytoplasmic activity. This finding, together with the fact that cycloheximide or 6-DMAP prevent pH_i oscillations, suggests that pH_i cycling might be tightly linked to the cdc2 kinase oscillations, which also represent a cytoplasmic activity depending on protein synthesis and phosphorylation.

When enucleation was performed at the metaphase II stage of meiosis, the SCWs recorded after egg activation were considerably lengthened, and, in some cases, were totally nonexistent. Sakai and Kubota (1981) were the first to notice that SCW periodicity was larger in anucleate fragments than in nucleate fragments, contrary to previous results (Hara et al., 1980), but interpreted this as resulting from differences in the procedures used to obtain anucleate fragments. However, Shinagawa (1983) clearly demonstrated that using either the method of Hara et al. (1980) or that of Sakai and Kubota (1981), the anucleate fragments still had an interval between two SCWs of longer duration than in nucleate fragments. However, in these two methods, an anucleate fragment is separated from the nucleate fragment long after fertilization, 30–45 min in the procedure of Sakai and Kubota (1981), 40–50 min in the procedure of Hara et al. (1980) (see a detailed comparison of the two methods in Shinagawa, 1983). The results presented here show that the periodicity of the SCWs is greatly increased if the delay between activation and enucleation is reduced. The SCW periodicity relative to control fertilized eggs was lengthened by only 6–15 min (39–48 min instead of 33–36 min) when enucleation was performed 30–50 min after fertilization (Shinagawa, 1983), whereas the SCW period is much more lengthened (35–60 min instead of 20–35 min), in fact almost doubled (mean value: 25 min in nucleated eggs vs. 44 min in enucleated eggs), when enucleation is performed at the time of activation (Table I). During the period between egg activation and enucleation, redistribution of nuclear material necessary for the correct control of SCWs may occur.

For the moment, the nature of the nuclear material that would be responsible for the synchronization between SCWs and the cell cycle oscillator (the cdc2 kinase oscillations) can be only a matter of speculation. In fact, most of the components of the germinal vesicle are dispersed into the cytoplasm during oocyte maturation, several hours before the metaphase II arrest. The most prominent difference between enucleating an egg at the metaphase II stage of meiosis (as in the present experiments) and an already activated egg (as in Shinagawa's experiments, 1983) is that the former procedure leads to the removal of the spindle, whereas in the latter the spindle dissociates after mitosis, and its components remain in the cytoplasm. It is therefore tempting to postulate that the nuclear material which is necessary for the correct timing of the SCWs, is either a spindle component or a protein associated with metaphasic chromosomes. The two experimental procedures used also lead to another difference: that of the presence or absence of a nucleus during the 30–50 min after activation. As early as 10–15 min after egg activation, the nucleus has resumed meiosis and become interphasic (Grandin and Charbonneau, 1989*a*) and DNA replication is then initiated (Kirschner et al., 1980). At least some of the nuclear components released into the cytoplasm after germinal vesicle breakdown could migrate into this interphasic nucleus, as has been observed for "early shifting" proteins that are accumulated by pronuclei soon after fertilization (Dreyer, 1987). When eggs are enucleated at the time of activation, this translocation of nuclear proteins cannot occur. In enucleated eggs, the deregulation of the SCWs could therefore also be due to the artefactual presence of these nuclear proteins in the cytoplasm.

In conclusion, we think that SCW cycling represents a basic oscillator, as first proposed by Kirschner and his coworkers (Hara et al., 1980; Kirschner et al., 1980; Gerhart et al., 1984; Kirschner et al., 1985; Kimelman et al., 1987), which needs redistribution of nuclear-associated material in order to be in phase with the cdc2 kinase oscillations. We have also shown that during the cell cycle of early embryos of *Xenopus*, pH_i cycling represents a true cytoplasmic activity in phase with MPF activity cycling. Its other characteristics, principally its dependence on protein synthesis and phosphorylation, make it resemble MPF activity which is also a pure cytoplasmic activity. Therefore, we propose that pH_i oscillations might be an integral component of the cell cycle oscillator. It will now be of interest to determine the hierarchy of control between the cdc2 kinase oscillations and pH_i oscillations.

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