Observations on Intracellular pH during Cleavage of Eggs of *Xenopus laevis*

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**Abstract**
Direct measurement of intracellular pH was made with recessed-tip pH microelectrodes in fertilized eggs of the frog, *Xenopus laevis*, from ~1 h after fertilization to mid-blastula. The intracellular pH just before first cleavage was 7.65 ± 0.04 (SD; n = 9). By stage 5 to the middle of stage 6, average intracellular pH was 7.70 ± 0.06 (SD; n = 16). A statistically significant alkalization of 0.18 ± 0.03 pH unit (SD; n = 5) was observed beginning in early blastula. A cycle of ≤0.05 pH unit was occasionally observed during the pre-blastula period, but its significance is unknown. By exposing the early cleavage embryo to saline buffered with sodium propionate, pH 4.7–5.0, it was possible to lower intracellular pH with some degree of control. Apparently, normal cleavage continued to occur when intracellular pH had been forced as much as 0.3 unit below normal. We conclude that this implies no specific involvement of intracellular pH in mitosis and cytokinesis. If intracellular pH was lowered further, cell division ceased at about pH 7.2, and furrow regression began at about pH 7.0. Once furrow regression occurred, subsequent development was usually arrested or abnormal when the embryo was transferred back to normal saline.

**MATERIALS AND METHODS**

**Procedures for Fertilized Eggs**

Adult *Xenopus laevis* were purchased from the South African Snake Farm (Fish Hoek, South Africa) or Nasco Biological Science (Fort Atkinson, Wis.). Some fertilized eggs were obtained from induced matings by the method of Guidon (10), but the majority of our material derived from in vitro fertilization. This was performed by exposing dry-stripped eggs to a sperm suspension in 20% Barth’s solution as described by Thoman and Gerhart (33). Fertilized eggs were dejellied in 8–10 min of gentle swirling in freshly-made 1.2% or 2.5% (wt/vol) cysteine HCl, pH 7.8–8.0. The dejellied eggs were washed, cultured, and recorded in Steinberg’s solution (20) or a modified Steinberg’s solution with HEPES buffer (MSSH). Steinberg’s solution contains NaCl, 60.0 mM; KCl, 0.7 mM; Ca(NO₃)₂, 0.3 mM; MgSO₄, 0.8 mM; Tris, 1.4 mM; pH 7.4; with HCl. The modified Steinberg’s contains NaCl, 57.7 mM; KCl, 0.7 mM; Ca(NO₃)₂, 0.3 mM; MgSO₄, 0.8 mM; HEPES, 5.0 mM; NaOH, 2.4 mM; pH 7.4. There was no difference, indicating an approximate doubling of the rate of protein synthesis from fertilization to two-cell stage, and another doubling from two-cell stage to blastula. Also, Graham and Morgan (8) have shown that it is not until mid-blastula that growth phases become an appreciable part of the embryonic cell cycle.

Changes of pH at this time might reflect the recruitment of different control mechanisms by the cell. We report that such a change may occur.

**Woodland** (39) on *Xenopus* and **Shih et al.** (27) on *Rana pipiens* indicate an approximate doubling of the rate of protein synthesis from fertilization to two-cell stage, and another doubling from two-cell stage to blastula. Also, Graham and Morgan (8) have shown that it is not until mid-blastula that growth phases become an appreciable part of the embryonic cell cycle.

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between the two solutions in any recorded embryonic property, and both supported development at least to hatching. All salts were reagent grade. L-Cysteine, Tris, HEPES, and, where used, MES (2-N-morpholinoethanesulfonic acid) and sodium propionate were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Experiment dish temperature was 19-22°C. Development of fertilized eggs was staged according to the Normal Table of Nieuwkoop and Faber (18).

Measurement of Intracellular pH

Measurements were made with conventional KCl and Thomas-type recessed-tip pH microelectrodes (34) as described previously (16). Conventional microelectrodes were filled with 3 M KCl, were typically of 20-25 Mohm resistance, and had been beveled (using a Sutter Instrument Model BV-10 beveler) to improve long-term stability. pH microelectrodes had a tip size of 0.5-1.0 μm, and the time required for full response to a unit pH change was 30-60 s. Electrode slopes were in the range 55-59 mV/pH unit. Recordings were made with the electrodes placed 200-300 μm apart in the animal hemisphere of an embryo confined in a depression of a plastic microtest dish (Falcon 3034; Falcon, Oxnard, Calif.).

Membrane potentials were recorded with a conventional electrophysiology preamplifier (design of P. Getting, University of Iowa), while the pH electrode went to an Analog Devices 311J varactor bridge electrometer (Analog Devices, Inc., Norwood, Mass.). Permanent records were made with a Linear Instruments Model 283 dual-channel chart recorder (Linear Instruments Corp., Irvine, Calif.) and Vetter Model A FM tape-recording system (A. R. Vetter Co., Rebersburg, Pa.). The indifferent electrode was normally provided by an agar bridge formulated in Steinberg's or MSSH and leading to a 3 M KCl reservoir, from which an Ag-AgCl wire was grounded. When very low pH variations of Steinberg's solution were employed, a standard glass calomel electrode (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) was used to ground the bath. For the most part, recordings were continuous through several cell divisions, the length of recording depending on electrode stability and the maintenance of good electrotonic coupling between the pH and conventional microelectrodes. Recordings were rejected if net drift of the electrodes exceeded 5 mV. Intracellular pH was generated electronically by subtracting the potential recorded by the conventional microelectrode from that of the pH microelectrode at the differential input of the chart recorder during playback of the tape-recorded experiment (as in Figs. 1 and 3), or manually from the real-time chart record (as in Fig. 2).

Although we usually initiated a measurement with both microelectrodes in the same animal blastomere, no effort was made to reposition them as they became separated by cell division. This assured continuity of recording while minimizing damage due to multiple impalement. For pH values obtained by this method to be accurate, the membrane potential seen by both electrodes must be the same at all times. Although equality of the membrane potential of blastomeres has not been demonstrated directly for early cleavage stages in Xenopus, evidence from later stages (30) and other amphibians (2, 13, 15) is strongly in favor of equipotential blastomeres. It has also been demonstrated that all surface cells on the animal hemisphere of the Xenopus embryo are electrotonically coupled at least into mid-blastula (20). When impalement of one cell of an embryo caused damage, this was reflected in depolarization of the other impaled cell, and the "healing" of each was similar (20, and our own observations).

RESULTS

Intracellular pH—Short-term Changes

Figs. 1 and 2 are examples of the continuous recording of intracellular pH during cleavage under normal conditions. The first five cleavages were the most useful to look for pH changes related to mitosis and cytokinesis, since the cycling of the cytokinesis-dependent membrane potential (E_m) makes it easy to gauge the period of the cell cycle. In most experiments, pH was almost constant during this period, and we could detect no changes of pH associated with either mitosis or furrowing (see Fig. 1). In two of seven continuous recordings of early cleavage stages, we observed a clear cycle of pH of ≤0.05 pH unit with the same period as the E_m cycle, but slightly out of phase with it (see Fig. 2). These pH changes do not appear to be passive since they are generally in a direction opposite to that which would occur if pH were influenced by E_m. In no experiment could we resolve an unequivocal pH cycle after stage 6.

Lowered Intracellular pH

To assess the importance of the pH cycle seen in Fig. 2 and the role, if any, of pH in cytokinesis, we decided to try to lower pH, 0.1-0.2 unit during cleavage. The Xenopus embryo is relatively insensitive to externally applied agents (28), and we similarly found a requirement for surprisingly strong treatments to effect the desired changes.

Our most successful procedure was to expose the embryo to a modified Steinberg's solution buffered at pH 4.7-5.0 with sodium propionate. (NaCl concentration was adjusted such that total Na remained near 60 mM.) Since the pK_a of pro-
concentrations seen by the embryo. Propionate concentrations may only approximate the actual initial concentrations because of incomplete mixing in the experiment dish, the stated initial pH is 4.73. In other experiments, pH would be forced to 7.0 or less by exposing the experiment dish to a short pulse of CO₂ gas. However, this procedure caused precipitous decreases of pH and was not useful for imposing smaller changes. On the whole, it was difficult to control pH in the range 7.0–7.3, even with propionate.

If the propionate was diluted out, or no further CO₂ was added, embryo pH would recover to normal values in 45–60 min. Where cleavage had been stopped, it would reinitiate when pH reached 7.2–7.5. However, regressed furrows rarely recovered, and subsequent development in those embryos which had suffered furrow regression was almost always abortive or abnormal. In one experiment in which pH was dropped to 6.46 with CO₂ before first cleavage, the recorded zygote and the two others in the experiment dish with it never cleaved, even when transferred back to normal MSSH.

Electrode impalement itself did not impair the development of normally reared embryos. Low extracellular pH and propionate itself have no effect inasmuch as embryos reared in propionate-containing Steinberg's solution are normal, even when transferred back to normal MSSH.

Intracellular pH—Long-term Changes

Table I lists the average pH of fertilized eggs through mid-blastula (stage 8). Fig. 2 is a typical example of the pattern we observed. The initial intracellular pH (measured 1 to 1.25 h after fertilization) was 7.65 ± 0.04 (SD; range 7.56–7.70) and rose slowly to 7.70 ± 0.06 at stage 5 to midway through 6 h.

**FIGURE 3** An example of a cleaving egg exposed to low pH, propionate-containing Steinberg's solution. This tape-playback record begins just at first cleavage. The egg was impaled before this, and initial pH was 7.63 in MSSH. Then a MES-buffered Steinberg's was added to the experiment dish to lower extracellular pH (pHₐ) to ~7.5. This dropped pHₐ ~0.1 unit. In other experiments, pHₐ would recover from this in a few minutes, but this embryo was further challenged with propionate before complete recovery. (a) Propionate-buffered Steinberg's solution was added to the experiment dish to a final propionate concentration of 5.4 mM; electrodes are in the same blastomere. (b) Early second cleavage, coincident with controls; electrodes now in separate cells. (c) Propionate added to 7.9 mM; from here on, pHₐ is nearly constant at 4.75. (d) Early third cleavage, with controls; to 9.1 mM propionate. (e) Early fourth cleavage, with controls; to 10.8 mM propionate. (f) To 12.3 mM propionate. (g) Early fifth cleavage, with controls. (h) Perhaps slight relaxation of the fifth cleavage furrow; controls are beginning sixth cleavage. (i) Embryo shows no change from h. At the end of the experiment, the fifth cleavage furrow is regressing; pHₐ is 4.73. Because of incomplete mixing in the experiment dish, the stated propionate concentrations may only approximate the actual initial concentrations seen by the embryo.
The early intracellular pH of 7.65 is almost exactly intermediate between the final pH of matured oocytes from hormonally stimulated and nonstimulated females (16). How this might relate to the hormonal history of egg donors is unclear because no uniform protocol was used in these experiments. Many of the donors had received no hormone for a number of months before induced ovulation, and oocytes from such donors tended to be variable (16). This contrasts with the reports of Webb and Nuccitelli (19) and Nuccitelli et al. (37) that pH; is ~0.3 unit less before fertilization. It is possible that the frog lowers pH; in the oviduct, as suggested by Smith and Ecker for Rana (31), or that in vitro maturation with progesterone does not accurately mimic all aspects of the in vivo process.

Mitosis and Cytokinesis

In the majority of our experiments, we were unable to detect any cycling of pH; with mitosis, and certainly nothing of the magnitude reported by Gerson and Burton (5) or Gillies and Deamer (6). In other experiments from this laboratory we were able to confirm a cycle of pH with mitosis in Physarum, although our values of pH; 7.0–7.4 with a peak just before mitosis differ considerably from the values reported by Gerson and Burton (M. Morisawa and R. A. Steinhardt, manuscript in preparation). This suggests that the pH; shifts observed in these other systems reflect changes associated with cell growth but not mitosis or cytokinesis per se. Gillies and Deamer suggest that increase in pH; are specifically related to DNA synthesis in Tetrahymena, but this would appear not to be the case in Xenopus, because pH; during meiotic maturation in the oocyte (16) can be as high as in the cleaving egg.

In two recordings we did observe a very small pH cycle, as has recently been reported by Webb and Nuccitelli (37) for early cleavage of Xenopus embryos which retain their jelly. Because these two recordings involved electrodes which had been partitioned into separate blastomeres by cell division, it is possible that the pH “cycle” is an artifact caused by the electrodes being in partially uncoupled cells with slightly different membrane potential cycling. In two control experiments we measured membrane potential with two conventional microelectrodes continuously over the period from before first cleavage to the middle of stage 6, and from stage 5 to stage 8. The membrane potentials measured by the electrodes were equal on the average and within ±1.5 mV of each other 90% of the time, but transient differences of up to 4 mV were observed. However, as far as we can tell, all variation between the electrodes was random, not cyclical, in nature. Hence, we cannot in our own data rule out the possibility of artifact producing the pH cycle; but it is just as reasonable to us that our inability to resolve a pH cycle more consistently could...
have been due to such random membrane potential variation obscuring a true pH oscillation.

In any case, it seems unlikely that distinct cytoplasmic events could be regulated by pH, changes as small as this. A possibility is that the change observed at the pH microelectrode reflects a localized pH change of greater magnitude in the deep cytoplasm. Arguing against this are those experiments in which the cytoplasm was acidified at artificially low pH values without disrupting cell division. It seems more likely that any pH change is a secondary effect related to the cycle of intracellular sodium activity (4, 29) or the insertion of highly K-permeable new membrane during furrowing (28, 40).

Neither did we observe anything to suggest a pH "trigger" for furrowing. If this were a localized change it might be missed, but the result was the same even when the pH electrode was very close to the developing furrow. Moreover, experiments in which the cytoplasm was acidified with propanoate as much as 0.3 pH unit below normal without affecting cytokinesis strongly suggests that pH is not involved in the regulation of this process.

The working hypothesis of many investigators of cytokinesis is that the contractile ring is analogous to a muscle sarcomere, including probable regulation by free Ca" levels (24). It is clearly true that treatments which increase membrane permeability to Ca" (7) or release it from intracellular stores (25) will induce surface contractions in frog eggs, as will the direct iontophoretic injection of Ca" but not Mg", K", Na", or CI"—immediately beneath the cell membrane (7).

However, other work involving the use of Ca" buffering agents in frog eggs (1, 22) and a mammalian cell model (3) has suggested that, while a certain threshold level of free Ca" is required for cytokinesis, this level is not measurably different from the probable resting level and need not change during cytokinesis. Efforts to detect increases in the Ca" level during cleavage with the Ca-dependent photoprotein aequorin have been inconclusive in frog and fish eggs (1, 21) and completely unsuccessful in the sea urchin egg (14). A second investigation of Ca" in the frog egg with a Ca-sensitive microelectrode also found no changes in Ca" levels (22).

Although other experiments suggest that Ca" may play a role in determining the site of the cleavage furrow (12, 35), it seems clear that Ca" does not regulate force production in the contractile ring as it does in the sarcosome. Moreover, the experiments reported here would appear to eliminate H", the last ion candidate for this regulator.

Lowering pH, to ~7.2 arrested cell division. The nature of this effect is unclear, although it may reflect a general repression of cellular metabolism and/or block mitosis. In amphibian embryos it has been demonstrated that low pH will uncouple electrotonically coupled cells in both Xenopus (36) and Ambystoma (32), and the pH required to effect this is similar to that needed to stop cleavage. However, it seems unlikely that uncoupling per se would disrupt cleavage because dissociated Ambystoma blastomeres continue to cleave (11).

Long-term Changes of Intracellular pH

We have observed two phases of alkalization in fertilized Xenopus eggs. The first of these occurs gradually during the first few divisions. It is quite small and may not be statistically meaningful. A change of pH reflecting the reported increase of protein synthesis at fertilization would be expected before first cleavage, as has been reported (19, 37).

The second increase in pH occurs in early blastula; it is dramatic and statistically significant. It suggests a correlation with the doubling of the rate of protein synthesis reported to occur between 2-cell stage and blastula. Although Shih et al. (27) do not describe the kinetics of this change in Rana, the Xenopus data of Woodland (39) imply that the change occurs after stage 6, which is consistent with our observations.

Membrane Potential Changes

The increase of E_m with development has been reported for a number of systems, most completely for Xenopus by Palmer and Slack (20). Our data are in good agreement with theirs except for stage 8, where our average value is lower. The cycling of E_m during early cleavage stages has also been reported (29). Slack and Warner (28) suggest that the increase of E_m during development is due to the increasing amount of new membrane facing on the developing blastocoel. The marked hyperpolarization midway through stage 6 to 7 would seem to reflect a more deliberate alteration of membrane and/or blastocoel properties. It might relate to the formation of the double-layered embryo in early blastula (18).

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