Reducing Inositol Lipid Hydrolysis, Ins(1,4,5)P₃ Receptor Availability, or Ca²⁺ Gradients Lengthens the Duration of the Cell Cycle in *Xenopus laevis* Blastomeres

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**Abstract.** We have microinjected an mAb specifically directed to phosphatidylinositol 4,5-bisphosphate (PIP₂) into one blastomere of two-cell stage *Xenopus laevis* embryos. This antibody binds to endogenous PIP₂ and reduces its rate of hydrolysis by phospholipase C. Antibody-injected blastomeres undergo partial or complete arrest of the cell cycle whereas the un.injected sister blastomeres divide normally. Since PIP₂ hydrolysis normally produces diacylglycerol (DG) and inositol 1,4,5-triphosphate (Ins[1,4,5]P₃), we attempted to measure changes in the levels of DG following stimulation of PIP₂ hydrolysis in antibody-injected oocytes. The total amount of DG in antibody-injected oocytes was significantly reduced compared to that of water-injected ones following stimulation by either acetylcholine or progesterone indicating that the antibody does indeed suppress PIP₂ hydrolysis. We also found that the PIP₂ antibodies greatly reduced the amount of intracellular Ca²⁺ released in the egg cortex during egg activation. As an indirect test for Ins(1,4,5)P₃ involvement in the cell cycle we injected heparin which competes with Ins(1,4,5)P₃ for binding to its receptor, and thus inhibits Ins(1,4,5)P₃-induced Ca²⁺ release. Microinjection of heparin into one blastomere of the two-cell stage embryo caused partial or complete arrest of the cell cycle depending upon the concentration of heparin injected. We further investigated the effect of reducing any [Ca²⁺]gradient by microinjecting dibromo-BAPTA into the blastomere. Dibromo-BAPTA injection completely blocked mitotic cell division when a final concentration of 1.5 mM was used. These results suggest that PIP₂ turnover as well as second messenger activity influence cell cycle duration during embryonic cell division in frogs.

A transient increase in cytoplasmic-free Ca²⁺ during the events of mitosis has long been recognized as an important regulatory step in many animal (1, 33, 34, 37, 49, 50, 56, 58, 60) and plant cells (25, 26, 36, 52, 66). Calcium stores have been found in close association with the mitotic apparatus in both animal and plant cells (see reference 63) and blocking the increase in intracellular Ca²⁺ by the injection of various Ca²⁺ chelators prevents mitotic events such as nuclear envelope breakdown, and consequently stops mitosis (34, 58, 60). This mitotic arrest was reversed by subsequent imposed increases in intracellular Ca²⁺ (58). It has also been shown that an artificial increase in intracellular Ca²⁺ generated by injecting Ca²⁺ buffers or Ins(1,4,5)P₃ caused premature chromatin condensation and the breakdown of the nuclear envelope in sea urchin embryos (60). There are also several reports that the Ca²⁺ receptor protein, calmodulin, is an important regulator of the cell cycle since calmodulin antagonists block progression of the cell cycle (6, 7). Thus, there is substantial evidence that [Ca²⁺], changes are required for normal mitotic events. Recently, periodic oscillations of the intracellular Ca²⁺ have been detected in dividing *Xenopus* embryos (23). The fact that many vertebrate embryos including *Xenopus* can divide in Ca²⁺ free media suggests that such oscillations in intracellular Ca²⁺ must indicate release from intracellular stores rather than Ca²⁺ influx across the plasma membrane. However the mechanism generating intracellular Ca²⁺ mobilization during cell division is unknown.

One well known [Ca²⁺] mobilization cascade begins with phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis (3), and a few studies implicate a possible role for the PIP₂ cycle in cell division. It has been shown that lithium inhibits mitosis in sea urchin zygotes and the application of exogenous

1. Abbreviations used in this paper: ACh, acetylcholine; DG, diacylglycerol; MBS, Modified Barth's Saline; MPF, maturation-promoting factor; MR, modified Ringers; NEB, nuclear envelope breakdown; PIP₂, phosphatidylinositol 4,5-bisphosphate.
myo-inositol reverses this lithium effect (18). Since lithium inhibits a phosphatase enzyme in the PIP2 metabolic pathway, these results suggest PIP2 cycling is necessary during mitosis of sea urchin zygotes. In addition, it has also been demonstrated that inositol triphosphates are produced after the sperm-induced Ca2+ transient declines, suggesting that Ins(1,4,5)P3 functions as a second messenger during the sea urchin cell cycle (9). Moreover recent studies indicate that PKC is an essential enzyme for the yeast cell cycle and support a possible role for the PIP2 cycle in cell cycle control (41).

Despite such suggestive evidence, the direct involvement of PIP2 hydrolysis in cell division remains obscure. In this study, we have microinjected two mAbs to PIP2, (namely ktlg and ktl0) into dividing blastomeres to two-cell stage Xenopus embryos. These PIP2 antibodies have previously been used to suppress PIP2 breakdown in at least three different cell types. The monoclonal anti-PIP2 antibody, ktlg, has been shown to abolish the mitogenic effect of PDGF and bombesin in NIH-3T3 cells (42). The introduction of this same antibody into yeast cells by electroporation also inhibited the mitotic cell cycle and this arrest was recovered by the application of mixtures of Ins(1,4,5)P3 and DG (61). Suppression of PIP2 breakdown by the antibody (kt10) inhibited the proliferation of ras-, src-, and erb-B transformed cells (19). Finally, PIP2 antibody blocked PDGF-mediated Ca2+ entry in rat vascular smooth muscle cells (31).

Here, we demonstrate that the microinjection of mAbs (ktlg, ktl0) to PIP2 into one blastomere of two-cell stage Xenopus embryos greatly lengthens the duration of the cell cycle of that blastomere while the sister blastomere cleaves normally. We find that the microinjection of PIP2 antibodies into Xenopus oocytes significantly reduces the cellular content of diacylglycerol (DG) in response to both acetylcholine and progesterone stimulation compared to that of control oocytes, confirming that the antibody reduces the amount of PIP2 hydrolysis. These same antibodies also greatly reduced the amount of Ca2+ released in the Xenopus egg cortex during activation process. Furthermore, suppressing Ins(1,4,5)P3-mediated intracellular Ca2+ release by injecting heparin inhibits the cell cycle in a dose-dependent manner. Finally, preventing intracellular Ca2+ gradients by dibromo-BAPTA injection suppresses mitotic cell division. These results strongly suggest that a cellular event downstream of PIP2 hydrolysis is crucial for the early mitotic cell cycle of Xenopus embryos.

Materials and Methods

Egg and Embryo Preparations

Adult Xenopus females were injected with 700-800 U of human chorionic gonadotropin (CG-10; Sigma Chemical Co., St. Louis, MO) into the dorsal lymph sac 8 h before experiments and maintained at room temperature (21-23°C). Eggs were stripped and fertilized with mixed testes of hypertonic Ringers (88 mM NaCl, 1.8 mM KCl, 2.4 mM NaHCO3, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 10 mM Na-Hepes; Sigma Chemical Co., pH 7.4). Individual oocytes at stage VI (15) were manually dissected from their outer follicles using watchmaker's forceps and stored in a 19°C incubator. Oocyte maturation was induced by the addition of 2 µM progesterone in MBS at room temperature.

Microinjection Procedures

Microinjection was performed using a Xenopus oocyte microinjector (Drummond Scientific, Broomall, PA). The micropipettes for injection were pulled on a vertical puller from 1.6 mm OD × 1.0 mm ID pyrex tubing (Drummond Scientific, Broomall, PA). The tip of the injection pipet was bevelled at a 45° angle (24) to minimize cell damage during injection. The injection volume was precalibrated by expelling the aqueous solution into oil and measuring the radius of this drop, although the Drummond microinjector has settings for volume to be injected. For each injection, errors were not >0.5% of the total volume of injection. For dibromo-BAPTA injection (usually <5 n1), we used Hiramoto's microinjection technique (28) which allows us to inject small volumes of solution with a great accuracy. Before and after each injection series, the injection volume was calibrated as described above. The final concentration of the injection solution in the embryo was calculated based upon the assumption that the actual accessible cytoplasmic volume of the 1.2 mm egg is 450 nl since half of the egg volume is estimated to be occupied by membrane-bound yolk platelets. All injections were made in 5% Ficoll/MBS for oocytes and 5% Ficoll/20% MR for embryos. Injected cells were then transferred to Ficoll-free solutions for culture. Poor cell cultures led to leakage of the cytoplasm and these embryos were discarded.

Solution Preparation

Oocytes and two-cell stage embryos were microinjected in their vegetal hemispheres with 30 nl of either PIP2 antibody (0.25-0.7 mg/ml), or various control agents: PBS (2.5×), mouse IgG (0.0 mg/ml; Sigma Chemical Co.) PIP2 antibody-PIP2 mixture (0.6 mg/ml of PIP2 antibody; PIP2 from Sigma Chemical Co.). The antibody was developed by immunizing mice with PIP2 prepared from bovine spinal cords, and a clone of hybridoma cells producing an antibody of immunoglobulin G2b class was purified (see reference 42 for detail). For PIP2 antibody-PIP2 mixture injection, ktlg was preincubated with liposomes containing PIP2:dimeristoylphosphatidylcholine:cholesterol (1:40-60, mole ratio) at room temperature for 2 h before microinjection. Approximately 80% of the ktl3g was absorbed with PIP2 in the condition where 100 times excess PIP2 was used in molar ratio (19, 42, 61). The stoichiometry of the antibody concentration to PIP2 concentration in the Xenopus embryo has not been determined. However, the amount of the antibody we injected (20-58 pmol) is estimated to be in excess of PIP2. Although no values are available for direct comparison in the Xenopus embryo, this was determined based upon the following assumptions. The PIP2 content in mammalian tissues ranges from 941-3190 pmol/mg of protein (8, 20, 46). If we assume that a similar level exists in the Xenopus embryo and there is ~2 µg of protein in the egg membrane (38), the concentration of PIP2 would be in the range of 4.7-16 pmol. Since the amount of the antibody we injected is 33-93 µg (20-58 pmol since IgG2b = 160 kD), we believe that most of the PIP2 is bound to the antibodies. Heparin (3 kD; Sigma Chemical Co.) injections and embryo handling followed the same procedure as above. Heparin was prepared at four different concentrations: 1.5, 0.75, and 0.37 mg/ml. The concentration of the DEAE-dextran coated heparin (15 kD; Sigma Chemical Co.) was 6 mg/ml. Dibromo-BAPTA (1,2-bis[2-bromino-5-(1,2-dihydro-3-oxo-2-propylidene)aminomethyl]-5-methoxy-2-naphthalenesulfonic acid (Molecular Probes, Eugene Oregon) solutions were prepared according to the equation (Ca2+)2 = Kd(Ca2+/dibromo-BAPTA)/(dibromo-BAPTA). The concentration of Ca2+ free dibromo-BAPTA in an 8-cell embryo ranged from 0.2 to 5.0 µM. 5-nl volumes were injected into a blastomere at two different sites that were far apart to speed equilibration in this large cell. Oocytes microinjected with 14C-glycerol were stored in an incubator at 19°C. All embryos were cultured at room temperature.
Figure 1. Cleavage inhibition induced by the microinjection of 30 nl of 0.5 mg/ml of anti-PIP2 antibody (kt10) into the blastomere of a two-cell stage embryo. Microinjections were made when the first cleavage furrow was completed. Large cells are descendents of the antibody-injected blastomere. The sister blastomere which was not injected with PIP2 antibody developed normally. A control embryo at the same age is shown at right. All embryos were cultured for 6 h before photographing them. Embryos are viewed looking down on the animal pole. Bar, 0.5 mm.

perature, washed with 20% MR three times, embedded in paraffin, sectioned at a thickness of 10 μm, and stained with bisbenzimide (Sigma Chemical Co.). Pictures were taken using a Nikon epifluorescence microscope (Nikon Inc., Garden City, NY).

Diacylglycerol Measurements
Manually defolliculated stage VI oocytes were microinjected with 50 nl of [14C]glycerol containing 0.1 μCi (NEN, NEC-046H Glycerol, [1,3-14C], 46.9 mCi/mmol). Radiolabeled oocytes were cultured in a 19°C incubator in an MBS containing 2.5 mM pyruvate, 400 μg/ml BSA, 10 μg/ml penicillin-G, 10 μg/ml streptomycin, 10 μg/ml gentamycin sulphate for 18–24 h. Once the oocytes exhibited steady-state labeling of the lipid fraction (18–24 h; see Fig. 4), either 30 nl of the PIP2 antibody or a control solution was microinjected into the cells. 10 min after microinjection, groups of six to nine oocytes were exposed to either 1 μM acetylcholine or 2 μM progesterone for 30 s followed by freezing in liquid N2. Control oocytes were neither injected nor exposed to any of these agents.

The lipid fraction was isolated as follows. Oocytes (six to nine) were homogenized by brief sonication in 1.5 ml polypropylene microcentrifuge tubes using 0.16 ml of deionized water followed by the addition of 0.6 ml of chloroform/methanol (1:2, vol/vol). Phases were separated after the addition of 0.2 ml of chloroform and 0.2 ml of deionized water. Organic phases were collected and aqueous phases were reextracted with 0.6 ml of chloroform. Both organic phases were collected and dried under N2 gas at room temperature. Samples were suspended in 0.1 ml of chloroform/methanol (2:1, vol/vol) and spotted onto the Silica Gel G thin layer chromatography plates (E. Merck, Darmstadt, Germany). Neutral lipids were dissolved in hexane/diethyl ether/acetic acid (70:30:3.5, vol/vol/vol) along with corresponding standards. Lipids were visualized with iodine vapor and DG bands were scraped to count radioactivity in a scintillation counter.

Results

Injection of the Anti-PIP2 Antibody into Xenopus Embryos at the Two-cell Stage
Microinjection of 30 nl of anti-PIP2 antibodies (kt3g, kt10) into one blastomere of two-cell stage embryos yielding a final concentration of 33–93 μg/ml noticeably retards cell division. Microinjection of larger dosages of antibodies (final concentration >130 μg/ml) completely stops division immediately and has a detrimental effect on later development. The cell cycle arrest and subsequent death of the embryo is not because of the direct effect of the high concentration of protein on the injected blastomere since microinjection of comparable amounts of nonspecific mouse IgG usually does not affect development. It seems more likely that cleavage arrest for a long period of time causes self destructive cytotoxic effects which ultimately result in the death of the embryos. Therefore we used a lower concentration which does not affect the viability of the embryos for most of this work.

The concentration of antibodies in the micropipette was 0.35–0.07 mg/ml for kt3g and 0.25–0.5 mg/ml for kt10, yielding a final cytoplasmic concentration range of ~33–93 μg/ml. We estimate that these concentrations are greater than that of PIP2 (see Materials and Methods). At these concentrations, the antibody-injected blastomere cleaved normally
for the next one or two cell cycles, but the subsequent cell cycles were greatly lengthened in duration as indicated by the large cell size (Fig. 1) and the less-densely populated, enlarged nuclei in comparison to the uninjected half of the embryo (Fig. 2). When embryos reached the late blastula stage, the descendant cells from the antibody-injected blastomere were comparable in size to those of embryos in the late morula or early blastula stage, and were much larger than cells descending from the noninjected sister blastomere (Fig. 1). Cytological examination indicated that the nuclei of the descendants of the antibody-injected blastomeres were prominently enlarged and nuclear plasms were loosely packed (Fig. 2 B), indicating chromosome condensation was inhibited. Antibody-injected embryos gastrulated abnormally, presumably because of the failure of invagination of large cells. The longer cell cycle duration was observed in almost all of the blastomeres descending from the injected blastomeres, although we often observed that the largest blastomeres were closest to the injection site, supporting the notion that those cells exhibited the longest cell cycles. The descendants from the uninjected blastomeres developed absolutely normally. The amount of cell cycle retardation observed in blastomeres injected with PIP2 antibodies is dose dependent within the range we have studied. At higher concentrations of antibodies, the size of daughter cells from antibody-injected blastomeres was larger than those injected with lower concentrations (data not presented).

Injection of Control Agents (PBS, Mouse IgG, and kt3g-PIP2) into Xenopus Embryos at the Two-Cell Stage

Microinjection of 30 nl of three control agents into one blastomere of a two-cell stage embryo had no significant effect on the cell cycle (Fig. 3). PBS was tested since PIP2 antibodies were prepared in it. Microinjection of PBS had the least effect on cleavage of the three control agents tested. The second control agent injected was nonspecific mouse IgG. The final concentration of mouse IgG for the data presented was 80 μg/ml. Concentrations as large as 133 μg/ml of mouse IgG had no significant effect on cell division, although at such high concentrations, we often observed small clusters of slightly larger, bulbous cells in the vicinity of the injection site. This swelling response was inconsistent, highly localized, and much smaller than the cell enlargement generated by the injection of PIP2 antibodies. While the injection of PIP2 antibodies caused the retardation of cell division resulting in the normal morphology of larger, younger blastomeres in all of the descendants of the injected blastomere, the injection of high concentrations of control IgG affected only a few of the descendants closest to the injection point and resulted in only a slight enlargement and rather abnormal morphology (bulbous shape) there alone. Lastly, microinjection of 30 nl of 0.6 mg/ml of kt3g which had been preincubated with PIP2 greatly decreased the effect of kt3g action as shown in Fig. 3. Like mouse IgG–injected blastomeres, injection of kt3g-PIP2 sometimes resulted in a cluster of slightly larger cells near the injection site. There is, however, a clear distinction between these cells and the descendants of PIP2 antibody-injected blastomeres. Some of the daughter cells from blastomeres injected with control agents were slightly larger than normal (usually bulging in shape and not at all like PIP2 antibody–injected cells which were severalfold larger) and were only found clustered near the injection site (whereas cell division retardation occurred over the entire half of the PIP2 antibody-injected embryos). Thus, these differences are probably a result of an artifact caused by the damage of microinjection or by a nonspecific effect of control agents.

Measurements of Diacylglycerol Changes in Oocytes

To determine that the injected antibody to PIP2 was indeed suppressing the rate of PIP2 hydrolysis, we directly mea-
Incorporation of the [14C]glycerol

Figure 4. Time course of the [14C]glycerol labeling of the total lipids in Xenopus oocytes. Stage VI oocytes were manually defolliculated and microinjected with 50 nl (0.5 μCi) of [14C]glycerol. After incubating at 19°C for the indicated time, groups of five oocytes were pooled and homogenized in 1 ml of Folch solution (CHCl3:CH3OH/2:1). Organic phases were collected and radioactivity was determined by scintillation counting. Results represent the mean values ± SD of three independent experiments.

We measured the DG levels in the Xenopus oocyte following the stimulation of PIP2 hydrolysis. We used the Xenopus oocyte instead of the Xenopus embryo because it is impossible to label the embryo to a steady-state level within the short period of 80–85 min between fertilization and first cleavage. The novel egg-labeling method originally described by Holwill et al. (30) was not successful for this experiment in several attempts mainly because of the death of the donor oocytes which had previously been labeled by microinjection with [3H]myo-inositol, matured in vitro, and transferred into a host female frog for jelly coat formation. Moreover, even if eggs were obtained it was very hard to get enough synchronously dividing cells so that at least five embryos could be pooled for each time point.

Two treatments that are known to stimulate PIP2 hydrolysis in Xenopus oocytes are the addition of the maturation-promoting hormone, progesterone (40, 62), and the neurotransmitter, acetylcholine (ACh) (29, 47). To determine the specific action of PIP2 antibody microinjection on PIP2 hydrolysis, changes of DG level were measured and compared in both ACh- and progesterone-stimulated stage VI oocytes, with and without injected PIP2 antibody. Stage VI oocytes labeled to steady state with [14C]glycerol (Fig. 4) were injected with 30 nl of water and treated 30 s with 1 μM ACh or 2 μM progesterone and exhibited an increase in DG production of 18.1 ± 9.7% and 47.0 ± 22.4%, respectively (Fig. 5). However, microinjection of 30 nl of 0.5 mg/ml of kit10 into oocytes blocked this increase in DG production following the addition of progesterone and actually reversed the ACh response slightly. This is good evidence that the antibody is indeed reducing the amount of PIP2 hydrolysis in response to these two treatments. The strong effects of the PIP2 antibodies on PIP2 hydrolysis are further supported by the reduction in Ca2+ release at activation in eggs that had been preinjected with PIP2 antibodies (Larabell and Nuccitelli, submitted for publication). Microinjection of PIP2 antibodies into the animal hemisphere of Xenopus eggs reduced the peak Ca2+ level achieved in the egg cortex during activation by ~50% compared to the same region of untreated eggs or eggs injected with an equal amount of nonspecific mouse IgG as a control. This result suggests that less Ins(1,4,5)P3 is being produced during activation in these PIP2 antibody-injected eggs.

Microinjection of Heparin into Two-cell Stage Xenopus Embryos

We have shown that Ins(1,4,5)P3 releases Ca2+ from the ER in Xenopus eggs (24) so we tested the hypothesis that the cell cycle requires changes in intracellular Ca2+ that would result from mobilization of Ins(1,4,5)P3. We injected heparin which is known to be a potent inhibitor of Ins(1,4,5)-P3-induced intracellular Ca2+ release by competing with Ins(1,4,5)P3 for binding to its receptor (17, 21, 64). It has been demonstrated that heparin inhibits Ins(1,4,5)P3-induced Ca2+-release from rat liver cells (27) and sea urchin egg homogenates (13), as well as intact sea urchin eggs (51) and we have observed the same inhibition in Xenopus eggs (Larabell and Nuccitelli, unpublished results). Microinjection of heparin into one blastomere of the two-cell stage Xenopus embryo inhibited cell division in a dose-dependent manner (Fig. 6). We have tested four different final concentrations of heparin, 200, 100, 50, and 25 μg/ml. The cell cycle was not affected by the injection of 25 μg/ml of heparin, but with a final concentration of 50 μg/ml of heparin, 14 out of 37 injected embryos exhibited a complete, immediate...
block of cell division in injected blastomeres and remained undivided through the next three to four rounds of cell division of their sister blastomeres. Subsequently, these injected blastomeres began dividing again but at a much slower rate than the uninjected blastomeres. The remaining 23 embryos injected with 50 μg/ml of heparin showed lengthened cell cycles without complete cleavage arrest. Uninjected control blastomeres underwent normal cell division. Blastomeres injected with a final concentration of 100 pg/ml of heparin stopped cell division for the next three or four cell cycles followed by slowed cell division in all of 33 injections. In 35 of 35 cases, 200 pg/ml of heparin completely blocked cell division as indicated by the large cell size of the descendants of the injected blastomere. As a control, we microinjected de-N-sulfated heparin which is not specific to Ins(1,4,5)P₃ binding sites. This did not inhibit cell division even at final concentrations as high as 400 μg/ml (Fig. 6 B).

Microinjection of Dibromo-BAPTA into Two Cell Stage Xenopus Embryos

This strong effect on the cell cycle by heparin suggests that Ins(1,4,5)P₃-induced Ca²⁺ release may be important for normal cell division. We further examined this possibility by suppressing intracellular [Ca²⁺] gradients. It has been shown that intracellular [Ca²⁺] gradients are greatly reduced by microinjecting into the cell the Ca²⁺-chelator, dibromo-BAPTA (1,2-bis[2-bis(carboxymethyl)amino-5-bromophenoxy]ethane)(57). Dibromo-BAPTA was used because it is among the most potent of the BAPTA buffers in suppressing [Ca²⁺], gradients required for fucus egg germination. We injected a mixture of dibromo-BAPTA:Ca²⁺-dibromo-BAPTA in a 3:1 ratio (free Ca²⁺ concentration of 0.4 μM) into one blastomere of two-cell stage Xenopus embryos. This mixture completely blocked cell division if a final cytoplasmic con-
The spatial distribution of the PIP2 which is important for cell cycle timing. One naturally thinks of a plasma membrane location for PIP2, but it has also been found in the nuclear envelope (5, 10, 11, 12). It is possible that the injected mAbs are interfering with PIP2 hydrolysis in the nuclear envelope and lengthening the cell cycle.

Microinjection of the inhibitor of Ins(1,4,5)P3-induced Ca2+ release, heparin, into one blastomere of two cell stage embryos either slowed or halted the cell cycle in a dose-dependent manner. Although heparin may have other cellular targets, it is the most widely used inhibitor of Ins(1,4,5)P3 binding to its receptor. The effective concentration range of 50–200 µg/ml is comparable to that used in previous studies (13, 27, 51). Our laboratory has confirmed that these levels of heparin greatly reduce Ins(1,4,5)P3-induced Ca2+ release in *Xenopus* eggs by using fura-2 fluorescence ratio imaging. These results demonstrate that heparin-sensitive Ins(1,4,5)P3 may play an important role during cell division by modulating intracellular Ca2+ levels. This agrees well with the previous finding that Ins(1,4,5)P3 functions as a second messenger during the cell cycle in sea urchins (9).

To confirm that intracellular (Ca2+) modulation is important for cell cycle control, we have artificially suppressed intracellular Ca2+ gradients by injecting the Ca2+ chelator, dibromo-BAPTA to shuttle Ca2+ from regions of high concentration to those of low concentration (57). Microinjection of a final concentration range between 0.5–1.5 mM dibromo-BAPTA either completely arrested or reduced the rate of cell division, depending upon the buffer concentration. Although we have not yet attempted to measure Ca2+ transients during cell division, these dibromo-BAPTA results imply that intracellular Ca2+ changes are required for mitotic cycling. In fact most recently, periodic oscillations of the intracellular (Ca2+) during cell division of *Xenopus* have been detected using Ca2+-selective microelectrodes (23). These findings agree well with numerous previous studies indicating a requirement for Ca2+ during mitotic processes in both plant and animal cells (1, 25, 26, 33, 34, 36, 37, 49, 50, 52, 56, 58, 60, 66) and support the involvement of Ins(1,4,5)P3-mediated Ca2+ release during the cell cycle. Although our data support a requirement for Ca2+ gradients during the cell cycle, this is still a controversial area because the detec-

### Table I. Dibromo-BAPTA Microinjection Slows *Xenopus* Blastomere Cleavage Rate

| Final conc. dibromo-BAPTA (mM) | Blastomeres exhibiting blocked cleavage | Blastomeres exhibiting slowed cleavage rates | Blastomeres exhibiting no change in cleavage rates |
|-------------------------------|----------------------------------------|------------------------------------------|-----------------------------------------------|
|                               | % (n)                                   | % (n)                                    | % (n)                                         |
| 2.0                           | 100 (4)                                 | 0 (4)                                    | 0 (4)                                         |
| 1.5                           | 100 (5)                                 | 0 (5)                                    | 0 (5)                                         |
| 1.0                           | 20 (5)                                  | 80 (5)                                   | 0 (5)                                         |
| 0.75                          | 0 (5)                                   | 60 (5)                                   | 40 (5)                                        |
| 0.5                           | 0 (4)                                   | 25 (4)                                   | 75 (4)                                        |

Inhibition of cell division by various concentrations of the Ca2+ chelator, dibromo-BAPTA. A mixture of dibromo-BAPTA: Ca2+-dibromo-BAPTA, in a 3:1 ratio (intracellular Ca2+ concentration = 0.4 µM, final concentration of dibromo-BAPTA = 0.5 to 2.0 mM, Kd of dibromo-BAPTA = 1.6 µM) was injected into one blastomere of two-cell stage embryos. Consecutive microinjections were performed at two different places in a blastomere to speed the equilibration of the antibody distribution. Injected embryos were checked for their division status every 10 min and were scored when control embryos reached the large cell size blastula stage.

To confirm that the injected antibody to PIP2 was indeed suppressing the rate of PIP2 hydrolysis, we measured DG levels and found that the normal increase in DG that follows progesterone or acetylcholine addition to immature oocytes is inhibited or even slightly reversed in PIP2 antibody-injected oocytes. These results suggest that the antibody suppresses PIP2 hydrolysis. This notion was further supported by the reduction in Ca2+ release at egg activation by PIP2 antibodies. Although one likely source of DG is from hydrolysis of PC, there are no reports to date that either progesterone or acetylcholine stimulate PC hydrolysis in *Xenopus* oocytes. In addition, the PIP2 antibodies have been shown to have no affinity toward other phospholipids including PC.

One interesting question that remains to be answered is what is the spatial distribution of PIP2 which is important for cell cycle timing. One naturally thinks of a plasma membrane location for PIP2, but it has also been found in the nuclear envelope (5, 10, 11, 12). It is possible that the injected mAbs are interfering with PIP2 hydrolysis in the nuclear envelope and lengthening the cell cycle.

The hydrolysis of PIP2 will generate increases in both Ins(1,4,5)P3 and DG. We have been unable to detect Ins(1,4,5)P3 in the frog egg using [3H]myo-inositol labeling or a radioimmunoassay kit (Amersham Corp., Arlington Heights, IL), but we present indirect evidence in support of its involvement in determining cell cycle duration. Microinjection of the inhibitor of Ins(1,4,5)P3-induced Ca2+ release, heparin, into one blastomere of two cell stage embryos either slowed or halted the cell cycle in a dose-dependent manner. Although heparin may have other cellular targets, it is the most widely used inhibitor of Ins(1,4,5)P3 binding to its receptor. The effective concentration range of 50–200 µg/ml is comparable to that used in previous studies (13, 27, 51). Our laboratory has confirmed that these levels of heparin greatly reduce Ins(1,4,5)P3-induced Ca2+ release in *Xenopus* eggs by using fura-2 fluorescence ratio imaging. These results demonstrate that heparin-sensitive Ins(1,4,5)P3 may play an important role during cell division by modulating intracellular Ca2+ levels. This agrees well with the previous finding that Ins(1,4,5)P3 functions as a second messenger during the cell cycle in sea urchins (9).

To confirm that intracellular (Ca2+) modulation is important for cell cycle control, we have artificially suppressed intracellular Ca2+ gradients by injecting the Ca2+ chelator, dibromo-BAPTA to shuttle Ca2+ from regions of high concentration to those of low concentration (57). Microinjection of a final concentration range between 0.5–1.5 mM dibromo-BAPTA either completely arrested or reduced the rate of cell division, depending upon the buffer concentration. Although we have not yet attempted to measure Ca2+ transients during cell division, these dibromo-BAPTA results imply that intracellular Ca2+ changes are required for mitotic cycling. In fact most recently, periodic oscillations of the intracellular (Ca2+) during cell division of *Xenopus* have been detected using Ca2+-selective microelectrodes (23). These findings agree well with numerous previous studies indicating a requirement for Ca2+ during mitotic processes in both plant and animal cells (1, 25, 26, 33, 34, 36, 37, 49, 50, 52, 56, 58, 60, 66) and support the involvement of Ins(1,4,5)P3-mediated Ca2+ release during the cell cycle. Although our data support a requirement for Ca2+ gradients during the cell cycle, this is still a controversial area because the detec-
tion of free Ca\(^{2+}\) changes during cleavage has proven to be quite difficult. In some cases even reductions of intracellular free Ca\(^{2+}\) during mitosis have been reported (35, 65). However, the most recent attempts using the aequorin technique for imaging intracellular Ca\(^{2+}\) have yielded positive results. In both fish and frog embryos a wave of free Ca\(^{2+}\) has been detected in the cleavage furrow region during cleavage. Perhaps it is this Ca\(^{2+}\) increase that our injections perturbed to lengthen the duration of the cell cycle.

While our data demonstrate an involvement of the PIP\(_2\) signal transduction cascade in the Xenopus embryonic cell cycle, it is not clear what might trigger this pathway. Unlike most receptor-mediated signal transducing mechanisms, the embryonic cell cycle requires neither ligand binding nor receptors. However, there are other studies which also implicate the inositol cascade in the cell cycle. The most relevant such study reported that microinjection of an antibody against a p\(^{20}\) H-ras onco-protein inhibited cell division in axolotl embryos (2) as well as in Xenopus embryos (43). This ras gene product is found predominantly on the cytoplasmic side of the plasma membrane and is a GTP-binding protein that activates various target enzymes, including PLC which hydrolyzes PIP\(_2\) in many systems including Xenopus oocytes (29). Furthermore, it has also been demonstrated that the microinjection of p\(^{20}\) ras into Xenopus oocytes induces meiotic cell division, and that maturation can be blocked by the injection of anti-ras antibody (14). However, it is not understood how ras itself is controlled. Recent studies on fission and budding yeasts have demonstrated that some gene products such as ste6 and cdc25, activate ras protein by promoting GDP-GTP exchange (4, 32, 54). This could imply the existence of a link between signal transduction pathways and cell cycle control mechanisms.

Maturation-promoting factor (MPF) is of fundamental importance in cell division and its concentration oscillates during the cell cycle. MPF is a protein kinase composed of a cyclin and p\(^{20}\) protein kinases, a homolog of a yeast cdc2 gene product (see reference 44 for review). In sea urchin embryos, inhibition of the synthesis of cyclin prevented the rise in intracellular [Ca\(^{2+}\)] indicating that MPF may be coupled to the Ca\(^{2+}\) transient during the sea urchin cell cycle (60). Furthermore, it has recently been shown that microinjection of a highly conserved sequence of p\(^{38}\)so2, called PSTAIR, triggers an increase in intracellular Ca\(^{2+}\) in both starfish and Xenopus oocytes. This further supports the notion that a component of MPF, p\(^{38}\)so2, interacts with an unknown cellular component of the Ca\(^{2+}\) regulatory system (48).

The control of cell cycle timing involves complex biochemical events whose complexity has begun to be uncovered by the recent progress on MPF studies. To understand more about cell cycle control mechanisms, it would be of great interest to elucidate the link between MPF activation and the cell signaling pathway such as oscillation of second messenger activities and that of MPF. This study indicates that PIP\(_2\) hydrolysis is necessary for the normal mitotic cell cycle in Xenopus embryos and that one of its products may function as a regulator of intracellular Ca\(^{2+}\) levels during cell division. Perhaps there is a causal link between this inositolpolyphosphate cycle regulation of Ca\(^{2+}\) and the oscillation of MPF activity.

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