Reducing Inositol Lipid Hydrolysis, $Ins(1,4,5)P_3$ Receptor Availability, or Ca^{2+} Gradients Lengthens the Duration of the Cell Cycle in *Xenopus laevis* Blastomeres

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Abstract. We have microinjected a mAb specifically directed to phosphatidylinositol 4,5-bisphosphate (PIP_2) into one blastomere of two-cell stage *Xenopus laevis* embryos. This antibody binds to endogenous PIP_2 and reduces its rate of hydrolysis by phospholipase C. Antibody-injected blastomeres undergo partial or complete arrest of the cell cycle whereas the uninjected sister blastomeres divide normally. Since PIP_2 hydrolysis normally produces diacylglycerol (DG) and inositol 1,4,5-triphosphate ($Ins[1,4,5]P_3$), we attempted to measure changes in the levels of DG following stimulation of PIP_2 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 PIP_2 hydrolysis. We also found that the PIP_2 antibodies greatly reduced the amount of intracellular Ca^{2+} released in the egg cortex during egg activation. As an indirect test for $Ins(1,4,5)P_3$ involvement in the cell cycle we injected heparin which competes with $Ins(1,4,5)P_3$ for binding to its receptor, and thus inhibits $Ins(1,4,5)P_3$-induced Ca^{2+} 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 $[Ca^{2+}]_i$ gradients 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 PIP_2 turnover as well as second messenger activity influence cell cycle duration during embryonic cell division in frogs.

A transient increase in cytoplasmic-free Ca^{2+} 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 Ca^{2+} by the injection of various Ca^{2+} 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 Ca^{2+} (58). It has also been shown that an artificial increase in intracellular Ca^{2+} generated by injecting Ca^{2+} buffers or $Ins(1,4,5)P_3$ caused premature chromatin condensation and the breakdown of the nuclear envelope in sea urchin embryos (60). There are also several reports that the Ca^{2+} 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 $[Ca^{2+}]_i$ changes are required for normal mitotic events. Recently, periodic oscillations of the intracellular Ca^{2+} have been detected in dividing *Xenopus* embryos (23). The fact that many vertebrate embryos including *Xenopus* can divide in Ca^{2+}-free media suggests that such oscillations in intracellular Ca^{2+} must indicate release from intracellular stores rather than Ca^{2+} influx across the plasma membrane. However the mechanism generating intracellular Ca^{2+} mobilization during cell division is unknown.

One well known $[Ca^{2+}]_i$ mobilization cascade begins with phosphatidylinositol 4,5-biphosphate (PIP_2)[1] hydrolysis (3), and a few studies implicate a possible role for the PIP_2 cycle in cell division. It has been shown that lithium inhibits mitosis in sea urchin zygotes and the application of exogenous

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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; PIP_2, phosphatidylinositol 4,5-biphosphate.

myo-inositol reverses this lithium effect (18). Since lithium inhibits a phosphatase enzyme in the PIP_2 metabolic pathway, these results suggest PIP_2 cycling is necessary during mitosis of sea urchin zygotes. In addition, it has also been demonstrated that inositol trisphosphates are produced after the sperm-induced Ca^{2+} transient declines, suggesting that $Ins(1,4,5)P_3$ 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 PIP_2 cycle in cell cycle control (41).

Despite such suggestive evidence, the direct involvement of PIP_2 hydrolysis in cell division remains obscure. In this study, we have microinjected two mAbs to PIP_2 (namely kt3g and ktl0) into dividing blastomeres to two-cell stage *Xenopus* embryos. These PIP_2 antibodies have previously been used to suppress PIP_2 breakdown in at least three different cell types. The monoclonal anti-PIP_2 antibody, kt3g, 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 $Ins(1,4,5)P_3$ and DG (61). Suppression of PIP_2 breakdown by the antibody (ktl0) inhibited the proliferation of ras-, src-, and erbB-transformed cells (19). Finally, PIP_2 antibody blocked PDGF-mediated Ca^{2+} entry in rat vascular smooth muscle cells (31).

Here, we demonstrate that the microinjection of mAbs (kt3g, ktl0) to PIP_2 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 PIP_2 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 PIP_2 hydrolysis. These same antibodies also greatly reduced the amount of Ca^{2+} released in the *Xenopus* egg cortex during activation process. Furthermore, suppressing $Ins(1,4,5)P_3$-mediated intracellular Ca^{2+} release by injecting heparin inhibits the cell cycle in a dose-dependent manner. Finally, preventing intracellular Ca^{2+} gradients by dibromo-BAPTA injection suppresses mitotic cell division. These results strongly suggest that a cellular event downstream of PIP_2 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 minced testis in 20% modified Ringers (1× MR: 100 mM NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Na-Hepes; Sigma Chemical Co., pH 7.8) and were then placed in 20% MR medium. At ~15 min after fertilization, embryos were dejellied in 2% cysteine hydrochloride (pH 7.8; Sigma Chemical Co.) for ~5 min and were then extensively washed in 20% MR. All embryos were raised in 20% MR at room temperature.

Oocytes Preparation

Mature female *Xenopus*, which had not been exposed to hCG during the previous four weeks, were anesthetized with either 3-aminobenzoic acid ethyl ester methane sulphonate salt (0.2% wt/vol; Sigma Chemical Co.) or by surrounding with ice, and sections of their ovaries were removed surgically into Modified Barth's Saline (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca[NO_3]_2$, 0.41 mM $CaCl_2$, 10 mM Na-Hepes; Sigma Chemical Co., pH 7.4). Individual oocytes at stage VI (15) were manually dissected from their outer follicles using watchmakers' 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 micropipets 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 impalement. 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 nl), 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 impalements caused 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 PIP_2 antibody (0.25–0.7 mg/ml), or various control agents: PBS (2.5×), mouse IgG (0.6 mg/ml; Sigma Chemical Co.), PIP_2 antibody-PIP_2 mixture (0.6 mg/ml of PIP_2 antibody; PIP_2 from Sigma Chemical Co.). The antibody was developed by immunizing mice with PIP_2 prepared from bovine spinal cords, and a clone of hybridoma cells producing an antibody of immunoglobulin G_{2b} class was purified (see reference 42 for detail). For PIP_2 antibody-PIP_2 mixture injection, kt3g was preincubated with liposomes containing PIP_2:dimyristoylphosphatidylcholine:cholesterol (1:40:60, mole ratio) at room temperature for 2 h before microinjection. Approximately 80% of the kt3g was absorbed with PIP_2 in the condition where 100 times excess PIP_2 was used in molar ratio (19, 42, 61). The stoichiometry of the antibody concentration to PIP_2 concentration in the *Xenopus* embryo has not been determined. However, the amount of antibody we injected (20–58 pmoles) is estimated to be in excess of PIP_2. Although no values are available for direct comparison in the *Xenopus* embryo, this was determined based upon the following assumptions. The PIP_2 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 ~5 μg of protein in the egg membrane (38), the concentration of PIP_2 would be in the range of 4.7–16 pmoles. Since the amount of the antibody we injected is 33–93 μg (20–58 pmoles since IgG2b = 160 kD), we believe that most of the PIP_2 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; 3, 1.5, 0.75, and 0.37 mg/ml. The concentration of the De-*N*-sulfated heparin (15 kD; Sigma Chemical Co.) was 6 mg/ml. Dibromo-BAPTA (1,2-bis[2-bis] [carboxymethyl]amino-5-bromophenoxy)ethane (Molecular Probes, Eugene Oregon) solutions were prepared according to the equation (Ca^{2+}) = Kd(Ca^{2+}-dibromo-BAPTA)/(dibromo-BAPTA) where kD = 1.6 μM. A mixture of dibromo-BAPTA:Ca^{2+}-dibromo-BAPTA in a 3:1 ratio gives final Ca^{2+} concentration of 0.4 μM (59). The final concentration of the Ca^{2+}-free form of dibromo-BAPTA in the embryo ranged from 0.5 to 2.0 mM. 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 ^{14}C-glycerol were treated in the same manner. Radio-labeled oocytes were stored in an incubator at 19°C. All embryos were cultured at room temperature.

Histology

Embryos were fixed in 4% paraformaldehyde, 20% MR for 6 h at room tem-

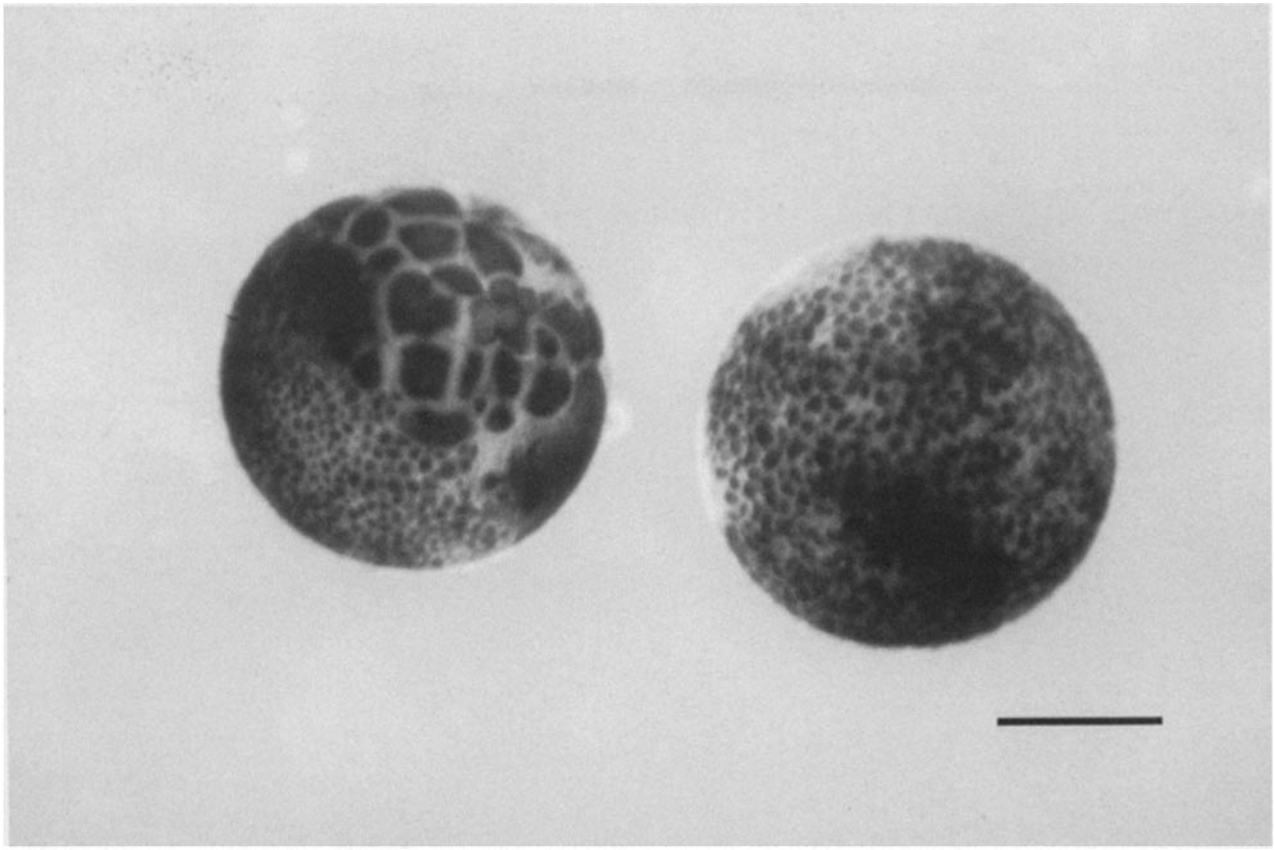


Figure 1. Cleavage inhibition induced by the microinjection of 30 nl of 0.5 mg/ml of anti-PIP_2 antibody (kt10) into the blastomere of a two-cell stage embryo. Microinjections were made when the first cleavage furrow was completed. Large cells are descendents of the antibody-injected blastomere. The sister blastomere which was not injected with PIP_2 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

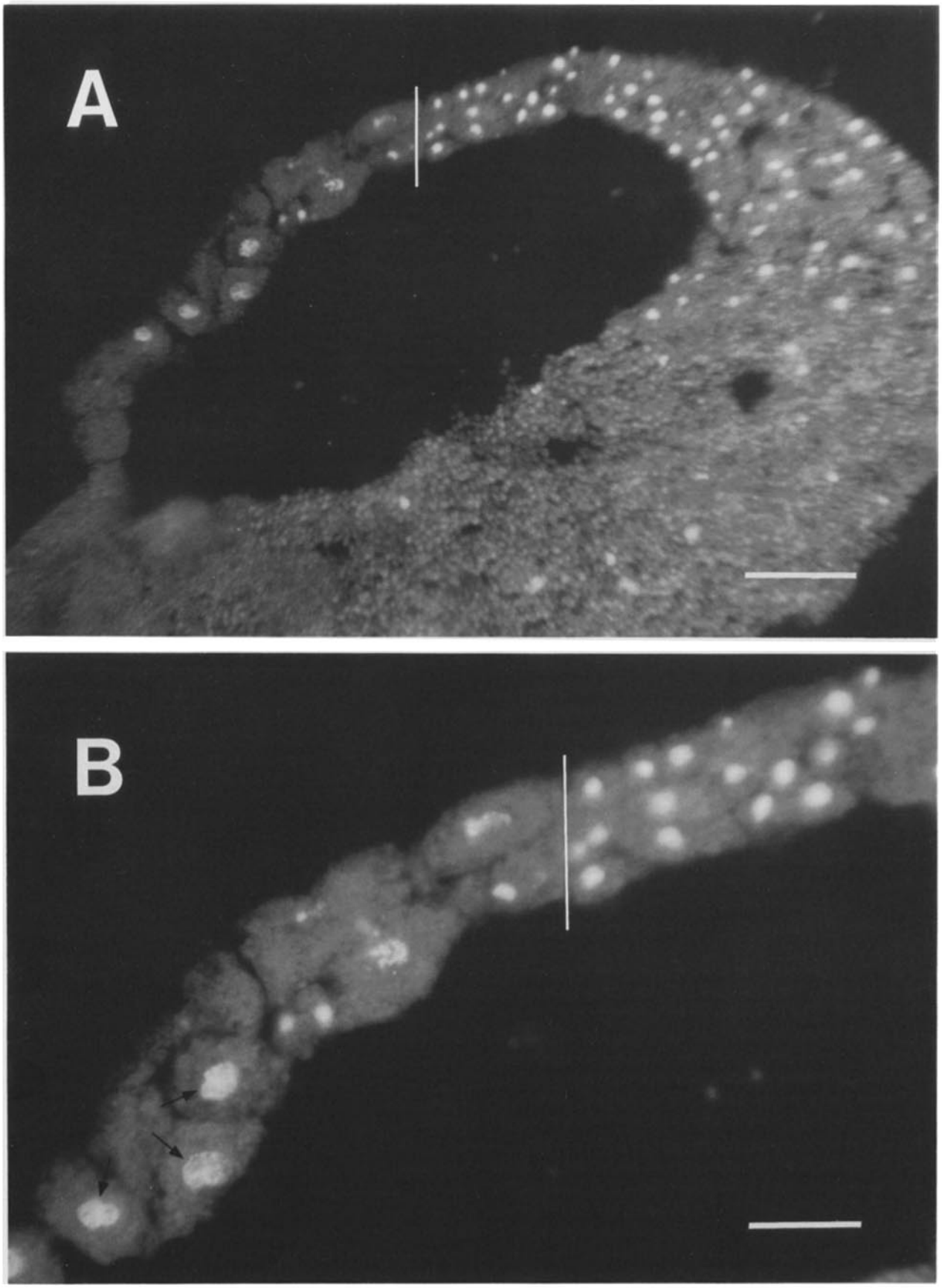
Manually defolliculated stage VI oocytes were microinjected with 50 nl of [^{14}C]glycerol containing 0.1 μCi (NEN, NEC-046H Glycerol, [1,3-^{14}C], 46.9 mCi/mmol). Radiolabeled oocytes were cultured in a 19°C incubator in an MBS containing 2.5 mM pyruvate, 400 μg/ml BSA, 10 μg/ml penicillin-G, 10 μg/ml 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 PIP_2 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 N_2. 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 N_2 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-PIP_2 Antibody into Xenopus Embryos at the Two-cell Stage

Microinjection of 30 nl of anti-PIP_2 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 0.35–0.7 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 PIP_2 (see Materials and Methods). At these concentrations, the antibody-injected blastomere cleaved normally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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 PIP_2 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-PIP_2) 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 PIP_2 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 PIP_2 antibodies. While the injection of PIP_2 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,

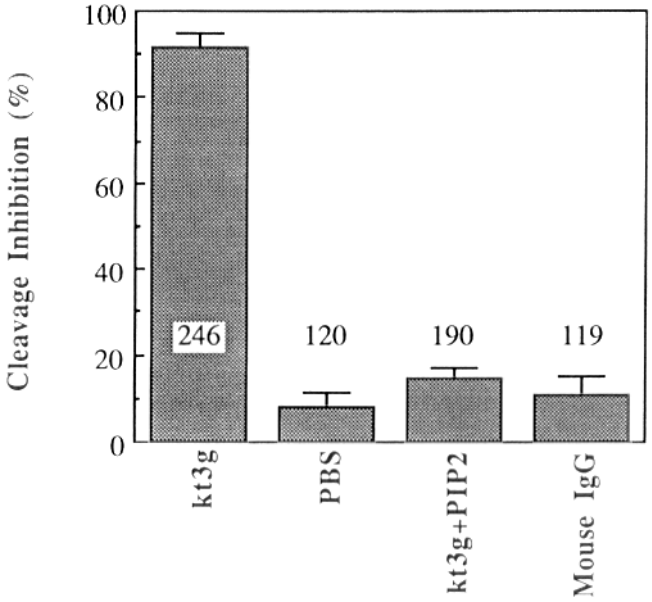

Figure 3. Effect of kt3g and control agents on cell division. One blastomere of a two-cell stage embryo was injected with either 30 nl of the PIP_2-antibody, kt3g (0.6 mg/ml), or with various control agents, PBS (2.5×), mouse IgG (0.6 mg/ml), and kt3g-PIP_2 (0.6 mg/ml) when the first cleavage furrow was ~50% completed. Five hours after injection, undamaged embryos were scored. Results represent the mean ± SD of six separate microinjection experiments for the kt3g and of four separate injection experiments for the controls. Numbers on or over bars represent the total number of embryos injected with the indicated substance.

microinjection of 30 nl of 0.6 mg/ml of kt3g which had been preincubated with PIP_2 greatly decreased the effect of kt3g action as shown in Fig. 3. Like mouse IgG–injected blastomeres, injection of kt3g-PIP_2 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 PIP_2 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 PIP_2 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 PIP_2 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 PIP_2 was indeed suppressing the rate of PIP_2 hydrolysis, we directly mea-

Figure 2. Fluorescence micrograph of a kt3g-injected *Xenopus* embryo. 30 nl of 0.6 mg/ml of kt3g were injected into one blastomere of a two-cell stage embryo and incubated for 6 h at 23°C. The embryo was then fixed in 4% paraformaldehyde in 20% MR, embedded in paraffin, sectioned to 10-μm-thick slices and stained with the DNA-specific dye, bisbenzimide. (*A*) Low magnification view showing the blastocoel cavity with a thin layer of ectodermal cells at the animal pole. The distribution of nuclei of the descendants from the antibody-injected blastomere (left of the line) was less dense than those of the uninjected half which developed normally. Antibody-exposed cells (left on the line) are much larger in size compared to unexposed cells and have greatly enlarged nuclei. (*B*) Higher magnification view of animal pole cells from *A*. Arrows indicate enlarged nuclei. Bars (horizontal): (*A*) 160 μm; (*B*) 40 μm.

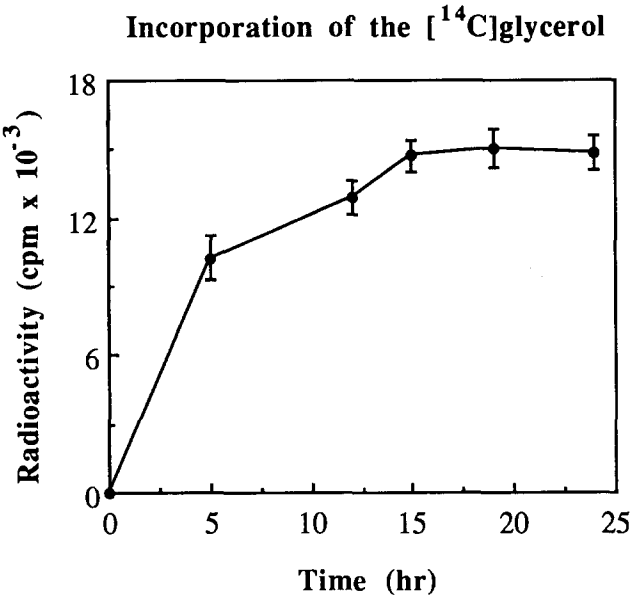

Figure 4. Time course of the [^{14}C]glycerol labeling of the total lipids in *Xenopus* oocytes. Stage VI oocytes were manually defolliculated and microinjected with 50 nl (0.5 μCi) of [^{14}C]glycerol. After incubating at 19°C for the indicated time, groups of five oocytes were pooled and homogenized in 1 ml of Folch solution ($CHCl_3$:CH_3OH/2:1). Organic phases were collected and radioactivity was determined by scintillation counting. Results represent the mean values ± SD of three independent experiments.

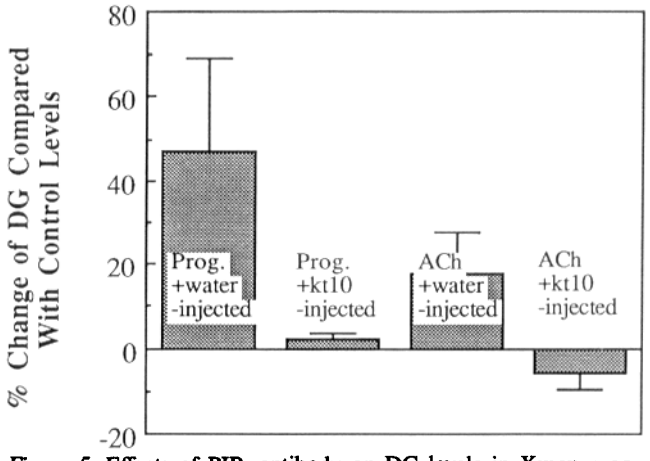

Figure 5. Effects of PIP_2 antibody on DG levels in *Xenopus* oocytes exposed to acetylcholine (1 μM) and progesterone (2 μM). Stage VI oocytes labeled to steady state with [^{14}C]glycerol were microinjected with 30 nl of 0.5 mg/ml kt10 or 30 nl of water. 10 min after injection they were transferred to medium containing either 1 μM ACh or 2 μM progesterone for 30 s. DGs were extracted and measured as described in Materials and Methods. The counts of ^{14}C-labeled DG were compared with those of control oocytes not exposed to either progesterone or ACh and the percent change was plotted. Error bars represent the mean ± SD of triplicate (*ACh + H_2O-injected, ACh + kt10-injected*) or duplicate (*Prog. + H_2O-injected, Prog. + kt10-injected*) experiments.

sured the DG levels in the *Xenopus* oocyte following the stimulation of PIP_2 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 [^{3}H]*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 PIP_2 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 PIP_2 antibody microinjection on PIP_2 hydrolysis, changes of DG level were measured and compared in both ACh- and progesterone-stimulated stage VI oocytes, with and without injected PIP_2 antibody. Stage VI oocytes labeled to steady state with [^{14}C]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 kt10 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 PIP_2 hydrolysis in response to these two treatments. The strong effects of the PIP_2 antibodies on PIP_2 hydrolysis are further supported by the reduction in Ca^{2+} release at activation in eggs that had been preinjected with PIP_2 antibodies (Larabell and Nuccitelli, submitted for publication). Microinjection of PIP_2 antibodies into the animal hemisphere of *Xenopus* eggs reduced the peak Ca^{2+} 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)P_3 is being produced during activation in these PIP_2 antibody-injected eggs.

Microinjection of Heparin into Two-cell Stage Xenopus Embryos

We have shown that Ins(1,4,5)P_3 releases Ca^{2+} from the ER in *Xenopus* eggs (24) so we tested the hypothesis that the cell cycle requires changes in intracellular Ca^{2+} that would result from mobilization of Ins(1,4,5)P_3. We injected heparin which is known to be a potent inhibitor of Ins(1,4,5)P_3-induced intracellular Ca^{2+} release by competing with Ins(1,4,5)P_3 for binding to its receptor (17, 21, 64). It has been demonstrated that heparin inhibits Ins(1,4,5)P_3-induced Ca^{2+}-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

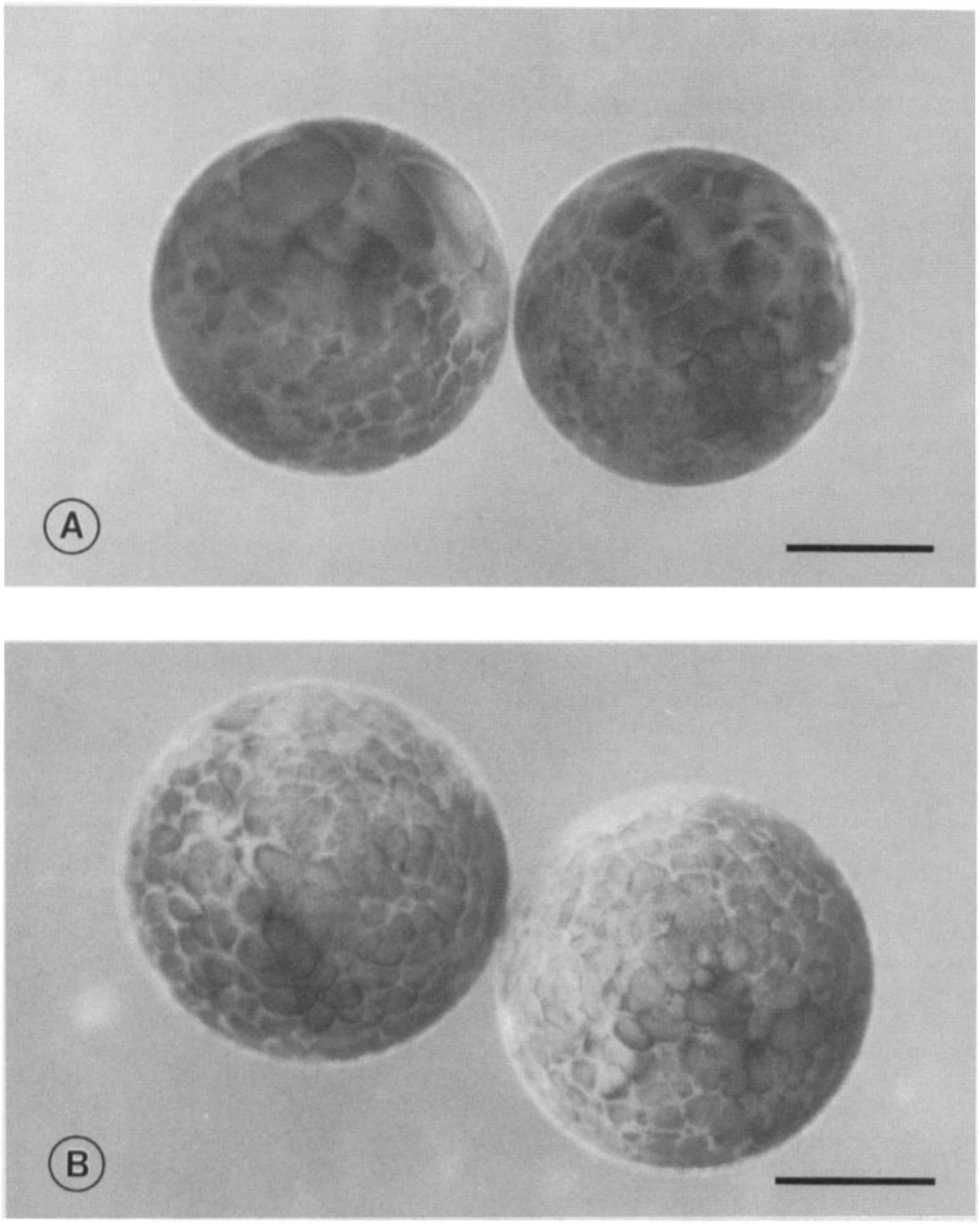

Figure 6. Effect of heparin and control de-*N*-sulfated heparin on cell division. (*A*) Microinjection of heparin (50 μg/ml) into one blastomere of two-cell stage embryos suppressed cell division in that blastomere as indicated by the large cell size of the descendants of the injected blastomere. (*B*) Control embryos injected at the same time with 400 μg/ml of de-*N*-sulfated heparin developed normally. Photos were taken when embryos were 4.5-h old. Embryos are viewed from the animal pole. Bar, 0.5 mm.

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 μg/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 μg/ml of heparin completely blocked the cell cycle and the injected blastomere remained undivided throughout the blastula stage. As a control, we microinjected de-*N*-sulfated heparin which is not specific to Ins(1,4,5)P_3 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)P_3-induced Ca^{2+} release may be important for normal cell division. We further examined this possibility by suppressing intracellular $[Ca^{2+}]_i$ gradients. It has been shown that intracellular $[Ca^{2+}]_i$ gradients are greatly reduced by microinjecting into the cell the Ca^{2+}-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 $[Ca^{2+}]_i$ gradients required for fucus egg germination. We injected a mixture of dibromo-BAPTA:Ca^{2+}-dibromo-BAPTA in a 3:1 ratio (free Ca^{2+} concentration of 0.4 μM) into one blastomere of two-cell stage *Xenopus* embryos. This mixture completely blocked cell divisic.ı if a final cytoplasmic con-

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

Final conc. dibromo-BAPTA	Blastomeres exhibiting blocked cleavage		Blastomeres exhibiting slowed cleavage rates		Blastomeres exhibiting no change in cleavage rates	
mM	%	(*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 Ca^{2+} chelator, dibromo-BAPTA. A mixture of dibromo-BAPTA: Ca^{2+}-dibromo-BAPTA, in a 3:1 ratio (intracellular Ca^{2+} 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.

centration of ≥1.5 mM was used. These injected blastomeres failed to cleave for up to at least three hours (approximately six cell cycles) while the sister uninjected blastomeres cleaved normally (Table I). The injection of dibromo-BAPTA resulting in a final cytoplasmic concentration below 0.5 mM had no effect on cell division (Table I). Concentrations between 0.5 and 1.5 mM lengthened the cell cycle time in a dosage-dependent manner as shown in Table I. At a concentration of 0.75 mM, none of five injected cells completely stopped cleaving but three out of five exhibited a longer cell cycle time and the remaining two divided normally.

Discussion

In this report, we have demonstrated that the injection of mAbs to PIP_2 into two-cell stage *Xenopus* embryos lengthens the duration of the cell cycle. The PIP_2 antibodies have been shown to be highly specific to PIP_2 and exhibit virtually no affinity for other phospholipids (PC, PE, PI) or Ins(1,4,5)P_3, but have weak affinity toward PIP (19, 31, 42, 61). The concentration range of 33–93 μg/ml did not affect the viability of the embryos, while inhibiting cleavage in a dose-dependent manner. We have not yet determined if the antibody lengthens a specific stage of mitosis. One likely target is nuclear envelope breakdown (NEB) since it has been shown that Ca^{2+} mobilization is required for NEB in the sea urchin egg (49, 58, 60) and swiss 3T3 fibroblasts (34). There is, however, no clear evidence for a close temporal correlation between Ca^{2+} transients and mitotic events. Time-lapse video analysis in conjunction with rigorous histological examination of the antibody-injected embryos would elucidate this issue.

To confirm that the injected antibody to PIP_2 was indeed suppressing the rate of PIP_2 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 PIP_2 antibody–injected oocytes. These results suggest that the antibody suppresses PIP_2 hydrolysis. This notion was further supported by the reduction in Ca^{2+} release at egg activation by PIP_2 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 PIP_2 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 the PIP_2 which is important for cell cycle timing. One naturally thinks of a plasma membrane location for PIP_2, but it has also been found in the nuclear envelope (5, 10, 11, 12). It is possible that the injected mAbs are interfering with PIP_2 hydrolysis in the nuclear envelope and lengthening the cell cycle.

The hydrolysis of PIP_2 will generate increases in both Ins(1,4,5)P_3 and DG. We have been unable to detect Ins(1,4,5)P_3 in the frog egg using [^{3}H]*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)P_3-induced Ca^{2+} 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)P_3 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)P_3-induced Ca^{2+} release in *Xenopus* eggs by using fura-2 fluorescence ratio imaging. These results demonstrate that heparin-sensitive Ins(1,4,5)P_3 may play an important role during cell division by modulating intracellular Ca^{2+} levels. This agrees well with the previous finding that Ins(1,4,5)P_3 functions as a second messenger during the cell cycle in sea urchins (9).

To confirm that intracellular (Ca^{2+}) modulation is important for cell cycle control, we have artificially suppressed intracellular Ca^{2+} gradients by injecting the Ca^{2+} chelator, dibromo-BAPTA to shuttle Ca^{2+} 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 Ca^{2+} transients during cell division, these dibromo-BAPTA results imply that intracellular Ca^{2+} changes are required for mitotic cycling. In fact most recently, periodic oscillations of the intracellular (Ca^{2+}) during cell division of *Xenopus* have been detected using Ca^{2+}-selective microelectrodes (23). These findings agree well with numerous previous studies indicating a requirement for Ca^{2+} 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)P_3-mediated Ca^{2+} release during the cell cycle. Although our data support a requirement for Ca^{2+} 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^{21} H-ras onco-protein inhibited cell division in axolotl embryos (2) as well as in *Xenopus* embryos (43). This ras gene protein 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 (22, 39, 45, 55). Transformed ras greatly affected the levels of second messengers generated by PIP_2 hydrolysis in many systems including *Xenopus* oocytes (29). Furthermore, it has also been demonstrated that the microinjection of p^{21} 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^{34} 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^{34cdc2}, 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^{34cdc2}, 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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References

1. Baker, P. F., and A. E. Warner. 1972. Intracellular calcium and cell cleavage in early embryos of *Xenopus laevis*. *J. Cell Biol.* 53:579–581.
2. Baltus, E., J. Hanocq-Quertier, F. Hanocq, and J. Brachet. 1988. Injection of an antibody against a p21 c-Ha-ras protein inhibits cleavage in axolotl eggs. *Proc. Natl. Acad. Sci. USA.* 85:502–506.
3. Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature (Lond.).* 341:197–205.
4. Broeck, D., T. Toda, T. Michaeli, L. Levin, C. Birchmeier, M. Zoller, S. Powers, and M. Wigler. 1987. The S. cerevisiae CDC25 gene product regulates the ras/adenylate cyclase pathway. *Cell.* 48:789–799.
5. Cataldi, A., S. Miscia, R. Lisio, R. Rana, and C. Cocco. 1990. Transient shift of diacylglycerol and inositol lipids induced by interferon in Daudi cells. Evidence for a different pattern between nuclei and intact cells. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 269:465–468.
6. Chafouleas, J. G., W. E. Bolton, H. Hidaka, A. E. Boyd III, and A. R. Means. 1982. Calmodulin and cell cycle: involvement in regulation of cell cycle progression. *Cell.* 28:41–50.
7. Chafouleas, J. G., L. Lagace, W. E. Bolton, A. E. Boyd III, and A. R. Means. 1984. Changes in calmodulin and its mRNA accompany reentry of quiescent (G0) cells into the cell cycle. *Cell.* 36:73–81.
8. Chilvers, E. R., I. H. Batty, R. A. J. Challiss, P. J. Barnes, and S. R. Nahorski. 1991. Determination of mass changes in phosphatidylinositol 4,5-bisphosphate and evidence for agonist-stimulated metabolism of inositol 1,4,5-trisphosphate in airway smooth muscle. *Biochem. J.* 275:373–379.
9. Ciapa, B., and M. Whitaker. 1986. Two phases of inositol polyphosphate and diacylglycerol production at fertilization. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 195:347–351.
10. Cocco, L., R. S. Gilmour, A. Ognibene, A. J. Letcher, F. A. Manzoli, and R. F. Irvine. 1987. Synthesis of polyphosphoinositides in nuclei of Friend cells. Evidence for polyphosphoinositide metabolism inside the nucleus which changes with cell differentiation. *Biochem. J.* 248:765–770.
11. Cocco, L., A. M. Martelli, R. S. Gilmour, A. Ognibene, F. A. Manzoli, and R. F. Irvine. 1988. Rapid changes in phospholipid metabolism in the nuclei of swiss 3T3 cells induced by treatment of the cells with insulin-like growth factor I. *Biochem. Biophys. Res. Commun.* 154:1266–1272.
12. Cocco, L., A. M. Martelli, R. S. Gilmour, A. Ognibene, F. A. Manzoli, and R. F. Irvine. 1989. Changes in nuclear inositol phospholipids induced in intact cells by insulin-like growth factor I. *Biochem. Biophys. Res. Commun.* 159:720–725.
13. Dargie, P. J., M. C. Agre, and H. C. Lee. 1990. Comparison of Ca^{2+} mobilizing activities of cyclic ADP-ribose and inositol trisphosphate. *Cell Reg.* 1:279–290.
14. Deshpande, A. K., and H. F. Kung. 1987. Insulin induction of *Xenopus laevis* oocyte maturation is inhibited by monoclonal antibody against p21 ras proteins. *Mol. Cell Biol.* 7:1285–1288.
15. Dumont, J. 1972. Oogenesis in Xenopus laevis (Daudin). I. Stages of oocytes development in laboratory maintained animals. *J. Morphol.* 136: 153–180.
16. Ferguson, J. E., J.-K. Han, J. P. Y. Kao, and R. Nuccitelli. 1991. The effects of inositol trisphosphates and inositol tetrakisphosphate on Ca^{2+} release and Cl^- current pattern in the *Xenopus laevis* oocyte. *Exp. Cell Res.* 192:352–369.
17. Ferris, C. D., R. L. Huganir, S. Supattapone, and S. H. Snyder. 1989. Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature (Lond.).* 342:87–89.
18. Forer, A., and P. J. Sillers. 1987. The role of the phosphatidylinositol cycle in mitosis in sea urchin zygotes. *Exp. Cell Res.* 170:42–55.
19. Fukami, K., K. Matsuoka, O. Nakanishi, A. Yamakawa, S. Kawai, and T. Takenawa. 1988. Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. *Proc. Natl. Acad. Sci. USA.* 85: 9057–9061.
20. Fukami, K., and T. Takenawa. 1989. Quantitative changes in polyphosphoinositides 1,2-diacylglycerol and inositol 1,4,5-trisphosphate by platelet-derived growth factor and prostaglandin $F_{2\alpha}$. *J. Biol. Chem.* 264:14985–14989.

21. Ghosh, T. K., P. S. Eis, J. M. Mullaney, C. L. Ebert, and D. L. Gill. 1988. Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. *J. Biol. Chem.* 263: 11075–11079.
22. Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick. 1984. Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. *Proc. Natl. Acad. Sci. USA.* 81:5704–5708.
23. Grandin, N., and M. Charbonneau. 1991. Intracellular free calcium oscillates during cell division of Xenopus embryos. *J. Cell Biol.* 112:711–718.
24. Han, J.-K., and R. Nuccitelli. 1990. Inositol 1,4,5-trisphosphate-induced calcium release in the organelle layers of the stratified, intact egg of *Xenopus laevis*. *J. Cell Biol.* 110:1103–1110.
25. Hepler, P. K. 1985. Calcium restriction prolongs metaphase in dividing *Tradescantia* stamen hair cells. *J. Cell Biol.* 100:1363–1368.
26. Hepler, P. K. 1989. Calcium transients during mitosis: observations in flux. *J. Cell Biol.* 109:2567–2573.
27. Hill, T. D., P.-O. Berggren, and A. L. Boynton. 1987. Heparin inhibits inositol trisphosphate-induced calcium release from permeabilized rat liver cells. *Biochem. Biophys. Res. Commun.* 149:897–901.
28. Hiramoto, Y. 1962. Microinjection of the live spermatozoa into sea urchin eggs. *Exp. Cell Res.* 27:416–426.
29. Hirono, C., I. Ito, and H. Sugiyama. 1987. Neurotensin and acetylcholine evoke common responses in frog oocytes injected with rat brain messenger ribonucleic acid. *J. Physiol.* 382:523–535.
30. Holwill, S., J. Heasman, C. R. Crawley, and C. C. Wylie. 1987. Axis and germ line deficiencies caused by u.v. irradiation of *Xenopus* oocytes cultured in vitro. *Development.* 100:735–743.
31. Huang, C.-L., T. Takenawa, and H. E. Ives. 1991. Platelet-derived growth factor-mediated Ca^{2+} entry is blocked by antibodies to phosphatidylinositol 4,5-bisphosphate but not involve heparin-sensitive inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.* 266:4045–4048.
32. Hughes, D. A., Y. Fukui, and M. Yamamoto. 1990. Homologous activators of ras in fission and budding yeast. *Nature (Lond.).* 344:355–357.
33. Izant, J. G. 1983. The role of calcium ions during mitosis: calcium participates in the anaphase trigger. *Chromosoma.* 88:1–10.
34. Kao, J. P., J. M. Alderton, R. Y. Tsien, and R. A. Steinhardt. 1990. Active involvement of Ca^{2+} in mitotic progression of swiss 3T3 fibroblasts. *J. Cell Biol.* 111:183–196.
35. Keith, C. H., F. R. Maxfield, and M. L. Shelanski. 1985. Intracellular free calcium levels are reduced in mitotic Pt K2 epithelial cells. *Proc. Natl. Acad. Sci. USA.* 82:800–804.
36. Keith, C. H., R. Ratan, F. R. Maxfield, A Bear, and M. L. Shelanski. 1985. Local cytoplasmic calcium gradients in living mitotic cells. *Nature (Lond.).* 316:848–850.
37. Kiehart, D. P. 1981. Studies on the in vivo sensitivity of spindle microtubules to calcium ions and evidence for a vesicular calcium-sequestering system. *J. Cell Biol.* 88:604–617.
38. Kline, D., G. S. Kopf, L. F. Muncy, and L. A. Jaffe. 1991. Evidence for the involvement of a pertussis toxin-insensitive G-protein in egg activation of the frog, *Xenopus laevis*. *Dev. Biol.* 143:218–229.
39. Lacal, J. C., S. K. Srivastava, P. S. Anderson, and S. A. Aaronson. 1986. Ras p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. *Cell.* 44:609–617.
40. Lacal, J. C., P. D. L. Pena, J. Moscat, P. Garcia-Barreno, P. S. Anderson, and S. T. Aaronson. 1987. Rapid stimulation of diacylglycerol production in *Xenopus* oocytes by microinjection of H-ras p21. *Science (Wash. DC).* 238:533–536.
41. Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner. 1990. A candidate protein kinase C gene, *PKC1*, is required for the cerevisiae cell cycle. *Cell.* 62:213–224.
42. Matuoka, K., K. Fukami, O. Nakanishi, S. Kawai, and T. Takenawa. 1988. Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. *Science (Wash. DC).* 239:640–643.
43. Miron, M.-J., J. Lanoix, and J. Paiement. 1990. Cytological effects of the microinjection of antibody to *ras* p21 in early cleavage *Xenopus* embryos. *Mol. Reprod. Develop.* 25:317–327.
44. Murray, A. W., and M. W. Kirschner. 1989. Dominoes and clocks: The union of two views of the cell cycle. *Science (Wash. DC).* 246:614–621.
45. Newmark, P. 1985. Events at the surface of the cell. *Nature (Lond.).* 317:380.
46. Nishihara, M., and R. W. Keenan. 1985. Inositol phospholipid levels of rat forebrain obtained by freeze-blowing method. *Biochim. Biophys. Acta.* 835:415–418.
47. Oron, Y., N. Dascal, E. Nadler, and M. Lupu. 1985. Inositol 1,4,5-trisphosphate mimics muscarinic response in *Xenopus* oocytes. *Nature (Lond.).* 313:141–143.
48. Picard, A., J.-C. Cavadore, P. Lory, J.-C. Bernengo, C. Ojeda, and M. Doree. 1990. Microinjection of a conserved peptide sequence of $p34^{cdc2}$ induces a Ca^{2+} transient in oocytes. *Science (Wash. DC).* 247:327–329.
49. Poenie, M., J. Alderton, R. Y. Tsien, and R. A. Steinhardt. 1985. Changes of free calcium levels with stages of the cell division cycle. *Nature (Lond.).* 315:147–149.
50. Poenie, M., J. Alderton, R. A. Steinhardt, and R. Y. Tsien. 1986. Calcium rises abruptly and briefly throughout the cell at the onset of anaphases. *Science (Wash. DC).* 233:886–889.
51. Rakow, T. L., and S. S. Shen. 1990. Multiple stores of calcium are released in the sea urchin egg during fertilization. *Proc. Natl. Acad. Sci. USA.* 87:9285–9289.
52. Ratan, R., M. L. Shelanski, and F. R. Maxfield. 1986. Transition from metaphase to anaphase is accompanied by local changes in cytoplasmic free calcium in ptk2 epithelial cells. *Proc. Natl. Acad. Sci. USA.* 83:5136–5140.
53. Rink, T. J., R. Y. Tsien, and A. E. Warner. 1980. Free calcium in *Xenopus* embryos measured with ion-selective microelectrodes. *Nature (Lond.).* 283:658–600.
54. Robinson, L. C., J. B. Gibbs, M. S. Marshall, and K. Tatchell. 1987. CDC 25: a component of the ras-adenylate cyclase pathway in *saccharomyces cerevisiae*. *Science (Wash. DC).* 235:1218–1221.
55. Shih, T. Y., A. G. Papageorge, P. E. Stokes, M. O. Weeks, and E. M. Scolnick. 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the $p21^{src}$ protein of harvey murine sarcoma virus. *Nature (Lond.).* 287:686–691.
56. Silver, R. B. 1989. Nuclear envelope breakdown and mitosis in sand dollar embryos is inhibited by microinjection of calcium buffers in a calcium-reversible fashion, and by antagonists of intracellular Ca^{2+} channels. *Dev. Biol.* 131:11–26.
57. Speksnijder, J. E., A. L. Miller, M. H. Weisenseel, T. H. Chen, and L. F. Jaffe. 1989. Calcium buffer injections block fucoid egg development by facilitating calcium diffusion. *Proc. Natl. Acad. Sci. USA.* 86:6607–6611.
58. Steinhardt, R. A., and J. Alderton. 1988. Intracellular free calciums rise triggers nuclear envelope breakdown in the sea urchin embryo. *Nature (Lond.).* 332:364–366.
59. Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry.* 19:2396–2404.
60. Twigg, J., R. Patel, and M. Whitaker. 1988. Translational control of $InsP_3$-induced chromatin condensation during the early cell cycles of sea urchin embryos. *Nature (Lond.).* 332:366–369.
61. Uno, I., K. Fukami, H. Kato, T. Takenawa, and T. Ishikawa. 1988. Essential role for phosphatidylinositol 4,5-bisphosphate in yeast cell proliferation. *Nature (Lond.).* 333:188–190.
62. Wasserman, W. J., A. B. Freedman, and J. J. LaBella. 1990. sn-1,2-Diacylglycerol levels increase in progesterone-stimulated *Xenopus laevis* oocytes. *J. Exp. Zool.* 255:63–71.
63. Whitaker, M., and R. Patel. 1990. Calcium and cell cycle control. *Development.* 108:525–542.
64. Worley, P. F., J. M. Baraban, S. Supattapone, V. S. Wilson, and S. H. Synder. 1987. Characterization of inositol trisphosphate receptor binding in brain. *J. Biol. Chem.* 262:12132–12136.
65. Yoshimoto, Y., T. Iwamatsu, and Y. Hiramoto. 1985. Cyclic changes in intracellular free calcium levels associated with cleavage cycles in echinoderm and medaka eggs. *Biomed. Res.* 6:387–394.
66. Zhang, D. H., D. A. Callaham, and P. K. Hepler. 1990. Regulation of anaphase chromosome motion in *Tradescantia* stamen hair cells by calcium and related signaling agents. *J. Cell Biol.* 111:171–182.