# Parthenogenetic activation decreases the polyphosphoinositide content of frog eggs

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Polyphosphoinositides were quantified in metaphase II-arrested eggs of the amphibian *Xenopus laevis*, and 8–10 min later in eggs activated by pricking. The content of phosphatidylinositol 4,5-biphosphate ($PIP_2$) was remarkably high in metaphase II-arrested eggs with respect to that of phosphatidylinositol 4-phosphate (PIP). It was found to drop dramatically at activation. In contrast PIP content did not change significantly.

*Polyphosphoinositide* *Phospholipase C activation* *Exocytosis* Xenopus *egg* *Parthenogenetic activation*

## 1. INTRODUCTION

How the egg is activated at fertilization is one of the central problems of cell biology. Since the pioneer work of Dalcq [1], the calcium theory of activation has received much experimental support, because eggs from many animal species can be parthenogenetically activated upon exposure to calcium-rich solutions [2], by microinjection of $Ca^{2+}$ buffers [3,4], or by the external application of the calcium ionophore A23187 [5–7]. In amphibians, activation can be induced simply by pricking metaphase II-arrested eggs with a needle [8]. The early events triggered by pricking are identical to those triggered by fertilization; in both cases, cortical granules fuse with the plasma membrane and release their contents outside the egg. This exocytosis causes elevation of an envelope (the fertilization membrane) around the egg, which provides a block to polyspermy. A wave-like propagation of contraction of the cortex, primarily in the animal hemisphere, occurs a few minutes after activation [9], and the second polar body is emitted about 30 min later. Cleavage however occurs only in fertilized eggs, unless a centriole is also introduced by pricking [8,10].

Activation by pricking requires the presence of external $Ca^{2+}$, which suggests that it is initiated by $Ca^{2+}$ leakage into the egg [11,12]. However, there is ample evidence that the main source of $Ca^{2+}$ required for activation is internal, in amphibians as in other animals [13]. In fact $Ca^{2+}$ entry from the medium seems to trigger the release of more $Ca^{2+}$ from intracellular stores. Recently evidence accumulated that inositol 1,4,5-triphosphate ($InsP_3$) might be involved in redistribution of intracellular $Ca^{2+}$ [14–17]. Actually $InsP_3$ microinjection was shown to induce activation when injected into both invertebrate and vertebrate eggs, including amphibians [18,19]. This led us to suspect that activation of phospholipase C might be involved as an early event in fertilization or parthenogenetic activation.

In agreement with this view, the present work shows that $PIP_2$, the substrate of phospholipase C in producing $InsP_3$, drops dramatically within a few minutes after the activation of *Xenopus* eggs by pricking.

## 2. MATERIALS AND METHODS

Stage VI *Xenopus laevis* oocytes (1.2–1.4 mm

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diameter) were isolated from an ovarian fragment, prepared free of follicle cells with watchmaker's forceps and incubated at 18°C in modified Ringer's (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA (pH 7.8) eventually containing 1 mCi/ml carrier-free [$^{32}$P]orthophosphate (Amersham, England). 4 h later, progesterone (1 $\mu$g/ml) was added, which induced meiotic maturation to progress to metaphase II. Eggs were released, or not, from metaphase II block by pricking them with a glass needle 14 h after starting the hormonal treatment. Eggs (activated or not) were fixed by transfer to plastic tubes containing boiling 7 mM $KH_2PO_4$ (10 eggs taken in 50 $\mu$l Ringer's injected into 500 $\mu$l $KH_2PO_4$ solution). After standing for 3 min at 95–100°C, the samples were rapidly frozen and kept at −20°C until use. To prepare the lipid extracts, the thawed samples were adjusted to 500 $\mu$l and briefly sonicated (10 s), then 1.5 ml of a methanol-chloroform mixture (2:1, v/v) was first added, followed by 0.5 ml of 2.4 N HCl, and finally 0.5 ml $CHCl_3$. Samples were thoroughly shaken, then centrifuged at 12000 × $g$ for 5 min. The lower phase was transferred into a plastic tube and the upper phase extracted again with 600 $\mu$l $CHCl_3$. After centrifugation, both lower phases were pooled, and washed with 1.2 ml of a mixture containing MeOH and 1 N HCl (1:1, v/v). The upper phase was discarded and the lower phase added to an equal volume of a solution containing 200 mM ammonium acetate in MeOH. The resulting mixture was applied to small columns (250 $\mu$l) containing neomycin bound to glass beads [20]. First, lipids other than the polyphosphoinositides were eluted with 150 mM ammonium acetate in $CHCl_3$-MeOH-$H_2O$ (3:6:1). Phosphatidylinositol 4-phosphate (PIP) was eluted with 600 mM ammonium acetate in $CHCl_3$-MeOH-$H_2O$ (3:6:1). Finally, $PIP_2$ was eluted with 1.5 N $NH_4OH$ in $CHCl_3$-MeOH-$H_2O$ (3:6:1). Aliquots of the PIP and $PIP_2$ fractions were counted in a liquid scintillation counter. To determine the chemical amounts of PIP and $PIP_2$ the fractions were first acidified (0.3 ml of 2.4 N HCl per ml PIP solution and 0.3 ml of 6 N HCl per ml PIP solution). After centrifugation, the upper phase was discarded and the lower phase was washed twice with 1 vol. of MeOH/1 N HCl (1:1). The lower phase was transferred into Pyrex tubes. After drying under nitrogen, 30 $\mu$l of 10% $Mg(NO_3)_2$ in 95% EtOH was added to each sample. The resulting material was mineralized, allowed to cool, then 0.3 ml of 0.5 N HCl was added. After standing for 15 min at 100°C, the samples were pooled and the amount of inorganic phosphate was determined [21]. The chemical amounts of PIP and $PIP_2$ were deduced from their phosphate content.

## 3. RESULTS

In initial experiments, $^{32}$P-preloaded *Xenopus* eggs were used to check the validity of the neomycin column procedure. As shown in fig.1, 3 clearcut peaks of radioactivity respectively

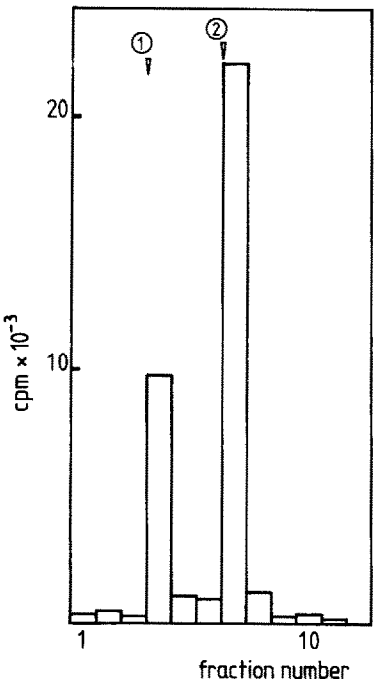


Fig.1. Elution profile of $^{32}$P-labelled PIP and $PIP_2$ from a neomycin column. A lipid extract (500 $\mu$l) was prepared from 10 *Xenopus* eggs preloaded with [$^{32}$P]orthophosphate, and passed through a small column containing neomycin bound to glass beads (250 $\mu$l beads). Lipids other than polyphosphoinositides were not retained on the neomycin column. PIP and $PIP_2$ were sequentially eluted (0.75 ml fractions) as described in section 2 by (1) 200 mM ammonium acetate, (2) 1.5 N ammonium hydroxide.

associated with non-polyphosphoinositides (not retained), PIP and $PIP_2$ could be separated. For each fraction, more than 90% of the total radioactivity was recovered in the first fraction (0.75 ml), and essentially complete elution was reached in the second fraction, thus before turning to the next eluting phase. Identification of the second and third fractions respectively with PIP and $PIP_2$, as well as purity of both fractions, was ascertained by TLC on EDTA-pretreated silica gel 60 using $CHCl_3$-MeOH-3.3 N $NH_4OH$ (43:38:12) as developing system, after desalting the eluted fractions, as reported in section 2 (not shown).

Next, *Xenopus* eggs arrested at second meiotic metaphase following progesterone treatment were activated, or not, by pricking them with a glass needle. Less than 4 min after pricking, a fertilization membrane elevated, and the activated eggs began to contract. They were fixed 8–10 min after pricking, when intensity of contraction was maximal, then polyphosphoinositide content was determined and compared with that of non-activated eggs. To circumvent possible egg variability, this experiment was performed twice (2 females) using 10 activated and 10 non-activated eggs.

As shown in fig.2, metaphase II-arrested eggs were found to contain a high amount of $PIP_2$ as compared with PIP (1.2 nmol $PIP_2$ per egg vs 0.07 nmol PIP). Following activation, $PIP_2$ level

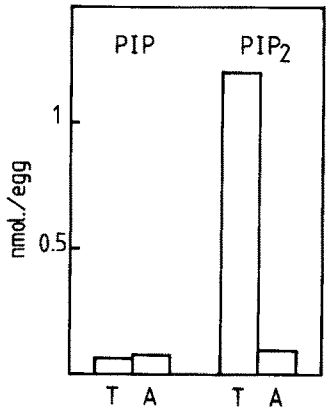


Fig.2. Comparison of the chemical amounts of PIP and $PIP_2$ before (T) and after (A) activation of *Xenopus* eggs. Ordinate: absolute amount of each compound per egg (10 eggs were used for each determination of chemical content).

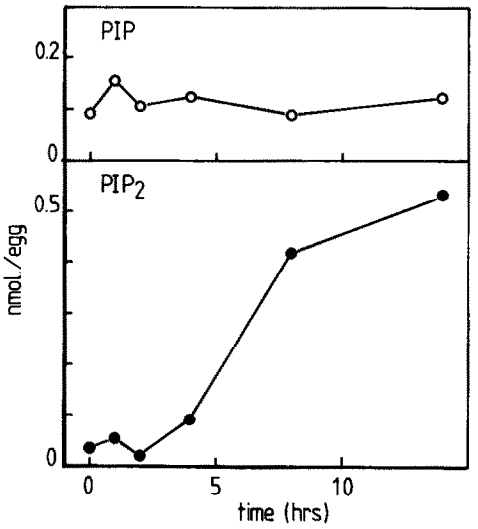


Fig.3. Variation in the concentrations of PIP and $PIP_2$ as a function of time after progesterone addition to prophase I-arrested oocytes.

dropped dramatically while PIP content remained unaltered. Essentially identical results were obtained in a separate experiment using $^{32}P$-preloaded eggs (not shown).

The unusually high amount of $PIP_2$ in metaphase II-arrested oocytes suggested that polyphosphoinositides might accumulate during meiotic maturation. Thus ovarian oocytes (i.e. oocytes arrested at first meiotic prophase) were isolated from another female and treated with progesterone (1 $\mu$g/ml) and the chemical amounts of PIP and $PIP_2$ were measured at various times of meiotic maturation (10 oocytes used for each determination). The white spot characteristic of germinal vesicle breakdown (GVBD) was observed in all hormone-treated oocytes within 4 h after progesterone addition. As shown in fig.3, polyphosphoinositide content increased after GVBD in maturing oocytes. While PIP content changed only slightly, $PIP_2$ content increased about 10-fold with respect to prophase I-arrested oocytes during meiotic maturation.

## 4. DISCUSSION

Here we show that $PIP_2$ content drops dramatically at the time of activation in *Xenopus* eggs, while PIP content does not change significantly. The implication is that $InsP_3$ must be

produced at that time. We showed previously that intracellular microinjection of 1 pmol $InsP_3$ is sufficient to trigger cortical granule exocytosis, cortical contraction and resumption of meiosis in *Xenopus* eggs [19]. From the present result it is obvious that the amount of $InsP_3$ originating from $PIP_2$ degradation at the time of activation is by far in excess compared to that required to induce these events. Thus our results provide strong experimental support for the view that $InsP_3$ acts as a second intracellular messenger to release intracellular $Ca^{2+}$ at the time of egg activation.

The intracellular store of $Ca^{2+}$ mobilized by $InsP_3$ is probably the cortical endoplasmic reticulum, since $InsP_3$ microinjection triggers cortical granules exocytosis only once this cellular compartment has differentiated, after progesterone treatment and in fact just before the egg arrests at second meiotic metaphase [19,22]. The resulting 'calcium explosion' [13] is probably responsible for the resumption of the cell cycle, since activity of the cytoplasmic factors controlling respectively spindle formation, chromosome condensation, and metaphase II arrest have been shown to be destroyed in vitro upon exposure to $Ca^{2+}$ [25–29].

The external $Ca^{2+}$ requirement for activation by pricking suggests that phospholipase C might be activated by $Ca^{2+}$ leakage into the egg. In agreement with this view, it was shown that micromolar $Ca^{2+}$ stimulates polyphosphoinositide hydrolysis in isolated cortices prepared from sea urchin egg [28].

Our results, as well as those reported by Whitaker and Aitchison [28] contrast with those of Turner et al. [29], which showed that the amounts of both PIP and $PIP_2$ increase in vivo upon activation of sea urchin eggs. Both results can be reconciled however if one considers that the main requisite for egg activation is $InsP_3$ production. Indeed, an increased $InsP_3$ production can result from: (i) the direct activation of phospholipase C; (ii) an increased production of its substrate.

Our results actually support the view that activity of phosphatidylinositol-4-phosphate kinase becomes very high after GVBD and prior to activation in *Xenopus* eggs. Thus, in amphibians, $PIP_2$ concentration would not be expected to be rate-limiting for phospholipase C activity in metaphase II-arrested eggs.

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