# Inositol 1,4,5-triphosphate microinjection triggers activation, but not meiotic maturation in amphibian and starfish oocytes

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Inositol 3,4,5-triphosphate (InsP3) brought about cortical granule exocytosis and elevation of a fertilization membrane, due to a rapid increase of free calcium in cytoplasm, when injected into oocytes of the amphibian *Xenopus laevis* arrested at second meiotic metaphase. The same result was observed when injection was performed into oocytes of the starfish *Marthasterias glacialis* arrested either at the first meiotic prophase or after completion of meiosis. Although meiotic maturation was induced in both animals by specific hormones which have been previously shown to release $Ca^{2+}$ within cytoplasm, InsP3 microinjection into prophase-arrested oocytes did not release them from prophase block.

*Inositol 1,4,5-triphosphate* *Calcium release* *Exocytosis* *Cell division*

## 1. INTRODUCTION

In most animals, full-grown oocytes are arrested in the ovary at the prophase stage of first meiotic division, with a large nucleus known as the germinal vesicle. In starfish as in amphibian, oocytes are released from prophase block by specific hormones (1-methyladenine in starfish, progesterone in amphibian), and they develop subsequently into fertilizable ova [1,2]. Several observations led to the suggestion that a hormone-induced increase in free calcium concentration may be a necessary first step to release both starfish and amphibian oocytes from prophase block [3–5].

On the other hand, increase in free $Ca^{2+}$ concentration has been shown to play a key role in fertilization or parthenogenetic activation in eggs of various animals, including amphibians and starfishes [6–10]. One of the earliest events of the activation process is the cortical reaction, during which vesicles fuse with the plasma membrane and secrete their content outside the plasma membrane. This exocytosis causes the elevation of an envelope (the fertilization membrane) around the egg, which provides a block to polyspermy.

Recently much evidence has accumulated from studies on permeabilized cells that InsP3 might be involved in redistribution of intracellular $Ca^{2+}$ [11–14]. This prompted us to examine the effects of InsP3 intracellular microinjection on both meiotic maturation and parthenogenetic activation.

We found that InsP3 microinjection readily induced cortical reaction in both amphibian and starfish oocytes, while it had no effect on the process of meiotic maturation.

## 2. MATERIALS AND METHODS

InsP3 was prepared from human erythrocyte $^{32}P$-labelled membranes essentially by the method of Downes et al. [15]. After incubation of the membranes with 1 mM $Ca^{2+}$, the membrane-free supernatant was applied to columns of AG1-X8



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resin (formate form) and the phosphate ester was eluted with appropriate ammonium formate/formic acid solution [16]. Column eluate was freed of $NH_4^+$ by treatment with AG50W-X8 resin (hydrogen form) and freeze-dried. This treatment was repeated a second time to reduce the amount of $Ca^{2+}$. The residue was neutralized with 10 N KOH. A sample (vehicle) was prepared under the same conditions, starting from the solution used to elute InsP3 from AG1-X8 resin. The concentration of InsP3 was calculated from its radioactivity and the specific radioactivity of its membrane precursor (phosphatidylinositol 4,5-biphosphate) which was determined in parallel after membrane extraction and phospholipid separation by TLC [17]. The authentication of InsP3 was controlled by paper chromatography in *n*-propanol/conc. $NH_3$/water (5:4:1) [18]. The final InsP3 solution (80 $\mu$M) has a $Ca^{2+}$ concentration of about 700 $\mu$M (determined by atomic absorption photometry), the same as that found in the vehicle sample. Before injections enough EGTA was added (from a 0.2 M solution at pH 7.0) to make the ratio (Ca total)/(EGTA total) = 0.5 in both the InsP3 stock solution and its vehicle. Free $Ca^{2+}$ was thus less than 0.6 $\mu$M, as checked with a $Ca^{2+}$-specific microelectrode [10].

Fully grown prophase-blocked oocytes of the starfish *Marthasterias glacialis* (160 $\mu$m diameter) were prepared free of follicle cells by washing them in $Ca^{2+}$-free seawater, as described in [19], then transferred in natural seawater before microinjections. Stage VI *Xenopus laevis* oocytes (1.2–1.4 mm) were isolated from an ovarian fragment and prepared free of follicle cells with watchmaker's forceps. The prophase-arrested oocytes were kept in modified Ringer's [0.1 M NaCl, 2 mM KCl, 1 mM Mg $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, 0.1 mM EDTA (pH 7.8)]. For injection into metaphase-arrested oocytes NaCl was replaced with KCl [20]. Microinjections were performed according to Hiramoto [21].

## 3. RESULTS

### 3.1. *Inositol 1,4,5-triphosphate microinjection triggers cortical granule exocytosis but not meiotic maturation in starfish oocytes*

Full-grown prophase-blocked oocytes of the starfish *glacialis* were injected with various amounts of InsP3. Assuming a homogeneous distribution within the oocyte and neglecting its possible degradation, the intracellular concentration of InsP3 ranged from 0.2 to 0.5 $\mu$M. All oocytes remained arrested at the prophase stage of meiosis. The injected oocytes were subsequently treated with $10^{-7}$ M 1-methyladenine (1-MeAde), less than 20 min after hormone addition, all InsP3-injected oocytes underwent germinal vesicle breakdown (GVBD). In fact the threshold concentration of 1-MeAde required for oocytes to be released from prophase block was not lowered in InsP3-injected oocytes (table 1). The duration of the hormone-dependent period (the period during which 1-MeAde is required in the medium for GVBD to occur) (3) was not decreased in InSP3-injected oocytes (not shown).

While InSP3 neither induced nor facilitated meiosis reinitiation, it readily triggered cortical granule exocytosis, even in prophase-blocked oocytes (similar results were observed in maturing and fully matured oocytes). When the intracellular concentration of InsP3 was 1.2 $\mu$M or higher, all microinjected oocytes elevated a beautiful fertilization membrane. The amount of InsP3 required to induce complete elevation of the fertilization membrane in 50% of the injected oocytes was about 0.8 $\mu$M. Finally, when the intracellular concentration of InsP3 ranged from 0.2 to 0.6 $\mu$M some oocytes were already activated, but the fertilization membrane elevated exclusively over the area of the plasma membrane near to the tip of the micropipette (table 1).

In another set of experiments, oocytes were first injected with 35 pl of 0.2 M EGTA solution (internal volume of an oocyte 2 nl). The EGTA-injected oocytes were subsequently microinjected with InsP3. Even when the intracellular concentration of InsP3 was as high as 5 $\mu$M, activation did not occur in such oocytes clamped at a low calcium level. Failure to undergo activation was not due to some unspecific and irreversible damage caused by EGTA microinjection, since the injected oocytes completed meiotic maturation upon subsequent addition of $10^{-6}$ M 1-MeAde. The possibility was also considered that impurities might have been concentrated with InsP3 in its preparation. Thus control experiments were performed by injecting 100 pl of the vehicle of InsP3 in its stock solution (see section 2). Control oocytes did not elevate a

Table 1

Effect of InsP3 microinjection on meiotic maturation and activation of starfish oocytes

| Oocyte pretreatment | InsP3 concentration in cytoplasm | Meiotic maturation | | Elevation of the fertilization membrane,[b] % |
|---|---|---|---|---|
| | | 1-MeAde, M | GVBD,[a] % | |
| | 0 | 0 | 0 | 0 |
| | | $5 \times 10^{-8}$ | 51 | 0 |
| | | $10^{-7}$ | 100 | 0 |
| | 5 $\mu$M | 0 | 0 (0/13) | 100 (13/13) |
| | | $5 \times 10^{-8}$ | 47 (6/13) | — |
| | | $10^{-7}$ | 100 (13/13) | — |
| | 2.5 $\mu$M | 0 | 0 (0/8) | 100 (8/8) |
| | | $10^{-7}$ | 100 (8/8) | — |
| None | 1.2 $\mu$M | 0 | 0 (0/8) | 100 (8/8) |
| | | $10^{-7}$ | 100 (8/8) | — |
| | 0.8 $\mu$M | 0 | 0 (0/9) | 44 (4/9) |
| | | $10^{-7}$ | 100 (9/9) | — |
| | 0.6 $\mu$M | 0 | 0 (0/10) | 30 (3/10)[c] |
| | 0.2 $\mu$M | 0 | 0 (0/9) | 11 (1/9)[c] |
| | | $10^{-7}$ | 100 (9/9) | — |
| Injected with 0.2 M EGTA (35 pl) | 5 $\mu$M | 0 | 0 (0/12) | 0 (0/12) |
| | | $10^{-7}$ | 100 (12/12) | 0 (0/12) |

[a] In parentheses: oocytes with GVBD/total number of InsP3 injected oocytes. Oocytes with GVBD were scored 1 h after InsP3 microinjection or 1 h after 1-MeAde addition. Results did not change later

[b] In parentheses: oocytes with elevated fertilization membrane/total number of InsP3-injected oocytes. Elevation of the fertilization membrane usually occurred within 3 min after efficient InsP3 injection, but results were scored 30 min later

[c] Oocytes with incomplete elevation of the fertilization membrane

fertilization membrane and they resumed meiotic maturation following addition of $10^{-7}$ M 1-MeAde.

### 3.2. *Inositol 1,4,5-triphosphate microinjection triggers parthenogenetic activation, but not meiotic maturation in* Xenopus *oocytes*

Stage VI prophase-blocked oocytes of the amphibian X. laevis were injected in the animal hemisphere with various amounts of InsP3. The final intracellular concentration of InsP3 ranged from 0.2 to 4 $\mu$M. Oocytes from the same batch were treated with 1 $\mu$g/ml progesterone. While the hormone-treated oocytes underwent GVBD about 8 h after progesterone addition, all the InsP3-injected oocytes remained arrested, even 24 h after InsP3 injection. However, the InsP3-injected oocytes seemed healthy, and most of them underwent GVBD upon subsequent progesterone addition (table 2).

In another set of experiments, oocytes were first treated with progesterone and InsP3 was injected only when they had reached metaphase II. It is well known that at this stage of development, amphibian eggs can be activated simply by pricking them with a glass needle [22]. However, activation by pricking can be avoided by replacing NaCl with KCl in the usual amphibian Ringer's solution [20]. Metaphase II-arrested eggs were transferred in such a medium and injected superficially in the animal hemisphere with 20 nl of 40 $\mu$M InsP3 solution (thus, the final intracellular concentration of

Table 2

Effect of InsP3 microinjection on meiotic maturation and activation of *Xenopus oocytes*

| Developmental stage of the recipient oocytes | InsP3 concentration in cytoplasm ($\mu$M) | Meiotic maturation[a] | Activation[b] |
|---|---|---|---|
| Prophase I-arrested | 0.2 | 0/20 | 0/20 |
| | 1 | 0/20 | 0/20 |
| | 4 | 0/20 | 0/20 |
| Metaphase II-arrested | 0[c] | | 4/20 |
| | 0.8 | | 20/20 |

[a] Number of oocytes with GVBD/number of injected oocytes. Results were scored 24 h after microinjection

[b] Number of oocytes (eggs) which elevated a fertilization membrane/number of microinjected cells. Results were scored 1 h after microinjection. They did not change later

[c] Eggs were injected with 20 nl of the vehicle of InsP3

InsP3 was about 0.8 $\mu$M). Cortical contractions occurred about 4 min after microinjection, then the vitellin membrane readily elevated in all InsP3-injected oocytes. About 30 min after InsP3 microinjection, the white spot at the animal pole disappeared and the second polar body was emitted. In control experiments injection of an identical volume of the vehicle of InsP3 did not trigger activation (table 2).

## 4. DISCUSSION

While this work was in progress, intracellular microinjection of InsP3 was shown to trigger cortical granule exocytosis and parthenogenetic activation in unfertilized sea urchin eggs [23]. This was an important result since it provided the first direct evidence that this compound can trigger $Ca^{2+}$ release in an intact non-permeabilized cell. Indeed, it has been clearly demonstrated that exocytosis occurs only if free $Ca^{2+}$ increases above 1 $\mu$M in the vicinity of the cortical granules [24]. Evidence that $Ca^{2+}$ is the intracellular effector of the cortical reaction includes its suppression in eggs injected with EGTA [10,25] and its induction in eggs treated with the $Ca^{2+}$ buffers of high free $Ca^{2+}$ content [26,27].

Here, the same result was obtained in two different groups of animals, including a vertebrate, and at different developmental stages. In starfish InsP3 microinjection triggered cortical granule exocytosis and elevation of the fertilization membrane, in prophase-arrested oocytes as well as in unfertilized eggs after completion of meiosis. In amphibian InsP3 trigered cortical granule exocytosis and parthenogenetic activation in metaphase II arrested but not in prophase I-arrested oocytes. In all cases, response to InsP3 was suppressed when cells were previously injected with a sufficient amount of EGTA, and the amount of InsP3 required to trigger exocytosis was lower than 1 $\mu$M (initial intracellular concentration).

1-MeAde, which triggers meiotic maturation in starfish has been shown to induce a transient increase of free $Ca^{2+}$ in cytoplasm of *M. glacialis* ([28], but see also [34]). In the same species, isolated cortices specifically release $Ca^{2+}$ upon hormone addition [29]. This led one to presume that $Ca^{2+}$ might be a second messenger in meiosis reinitiation of starfish oocytes. In this study InsP3 was injected over a wide range of concentrations in oocytes responsive to 1-MeAde. Although free $Ca^{2+}$ increased in the cortical region, since cortical granule exocytosis occurred when the amount of microinjected InsP3 was sufficient, meiosis reinitiation was neither induced nor facilitated. Cell fractionation experiments indicate the InsP3-sensitive release site is probably restricted to the en-

doplasmic reticulum [30], while ionophore A23187 also releases $Ca^{2+}$ from mitochondria [31]. Like InsP3 ionophore A23187 has no effect on meiosis reinitiation in starfish [9].

Therefore, meiotic maturation in starfish is probably not dependent upon an increase in free calcium concentration. A previous study on the effects of injecting EGTA and $Ca^{2+}$ buffers of increasing p*Ca* led to the same conclusion [27].

InsP3 microinjection was also not found to induce meiotic maturation in amphibian oocytes. This was an unexpected result since external application of ionophore A23187 [7,8] and superficial $Ca^{2+}$ iontophoresis in the animal hemisphere [32] have been shown to induce meiotic maturation in the *Xenopus* oocyte. Efficacy of both treatments probably depends on an increase of free $Ca^{2+}$ concentration in the cortical region of the oocyte. Although free $Ca^{2+}$ certainly increases near the cortical granules in response to InsP3 in metaphase II-arrested oocytes, since it triggers exocytosis, this might not be the case in prophase I-arrested oocytes. As a matter of fact cortical endoplasmic reticulum forms only several hours after progesterone treatment in *Xenopus* oocytes [33]. Therefore the possibility must be considered that localization of the InsP3-sensitive $Ca^{2+}$ stores in prophase-arrested *Xenopus* oocytes prevents InsP3 being an efficient inducer of meiotic maturation. The implication would be that the transient increase of free $Ca^{2+}$ after progesterone application, which presumably occurs in the cortical region of the *Xenopus* oocyte [5], does not originate from the endoplasmic reticulum.

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