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Phospholipase C-dependent Ca^{2+} release by worm and mammal sperm factors☆

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Abstract

Egg activation in all animals evidently requires the synthesis of inositol 1,4,5-trisphosphate ($InsP_3$) from phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). Depending on the organism, $InsP_3$ elicits either calcium oscillations or a single wave, which in turn initiates development. A soluble component in boar sperm that activates mammalian eggs has been suggested to be a PLC isoform. We tested this hypothesis in vitro using egg microsomes of *Chaetopterus*. Boar sperm factor elicited Ca^{2+} release from the microsomes by an $InsP_3$-dependent mechanism. The PLC inhibitor U-73122, but not its inactive analog U-73343, blocked the response to sperm factor but not to $InsP_3$. U-73122 also inhibited the activation of fertilized and parthenogenetic eggs. *Chaetopterus* sperm also contained a similar activity. These results strongly support the hypothesis that sperm PLCs are ubiquitous mediators of egg activation at fertilization.

Egg activation at fertilization results from one or more waves of intracellular Ca^{2+} release [1–3]. This release originates from intracellular calcium stores [4–6] and is elicited by one or more intracellular second messengers. These include inositol 1,4,5-trisphosphate ($InsP_3$), cyclic adenosine diphosphate ribose (cADPR), and nicotinate adenine dinucleotide phosphate (NAADP). The receptors for $InsP_3$ and cADPR are on the endoplasmic reticulum (ER) [7]; those for NAADP are on lysosomal-related organelles [8]. Of these three, cADPR and NAADP release Ca^{2+} in sea urchin eggs and homogenates [9–12], but not those of most other organisms [2]. However, all animal eggs apparently utilize $InsP_3$ in their Ca^{2+} release at fertilization [13–17].

The biochemical processes leading to $InsP_3$ synthesis and Ca^{2+} release at fertilization have been controversial. Two general classes of hypotheses, involving either sperm–egg contact or fusion, differ in that contact-based hypotheses suggest that sperm activate eggs by binding to receptors and activating intracellular signal transduction systems similar to those in somatic cells [18], whereas fusion-based hypotheses suggest that sperm introduce some soluble substance into the egg that directly activates some step in the signal transduction process [11,18].

While there is evidence to suggest that sperm can activate eggs of at least one species by contact [19], more recent research has been directed at soluble sperm factors that could be introduced into the egg after fusion. The sperm factor hypothesis is based on the observations that microinjection of sperm [20] or soluble extracts from sperm but not other tissues can elicit Ca^{2+} responses characteristic of fertilization and activate eggs [21–23]. Such activity is apparently widespread in the animal kingdom, being found in vertebrates [22,24,25], non-vertebrate chordates [26], and nemertean worms [21].

Since mammalian sperm have phospholipase C (PLC) activity that is capable of synthesizing $InsP_3$ [15], it was

☆ *Abbreviations:* CaFASW, calcium-free artificial seawater; cADPR, cyclic adenosine diphosphate ribose; $InsP_3$, inositol (1,4,5)-trisphosphate; NAADP, nicotinate adenine dinucleotide phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

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suspected that the active agent in sperm factor might be a PLC. However, initial studies indicated that no known isoform of PLC was the active component either in vivo or in sea urchin egg homogenates [27]. An alternative approach, which involved searches for PLC-related ESTs in mouse, human, and monkey testes, identified a novel phospholipase C, PLCζ, that could activate egg Ca^{2+} oscillations and development in vivo and Ca^{2+} release in sea urchin egg homogenates [13,28].

Because they can respond to additional second messengers that play only a marginal role in mammalian eggs, and in addition display only a single calcium transient at fertilization, sea urchin egg homogenates may not be an appropriate model for egg activation in mammals. Here we have used egg microsomes of *Chaetopterus* to test whether or not PLC in sperm factor is necessary and sufficient to elicit Ca^{2+} release. Fertilized *Chaetopterus* eggs, like those of mammals, undergo Ca^{2+} oscillations in response to fertilization [29]. Also like mammal eggs, *Chaetopterus* egg microsomes can release Ca^{2+} only in response to $InsP_3$ [30]. Therefore, if the active component of sperm factor is a PLC, inhibition of PLC activity should prevent mammalian sperm factor from releasing Ca^{2+} in this system.

Accordingly, we tested whether or not the PLC inhibitor, U-73122, could prevent mammalian sperm factor from releasing Ca^{2+}. We found that it could. In addition, the same inhibitor blocked egg activation by sperm. Protostome eggs can also be activated by exposure to excess K^+. It had been long presumed that the mechanism of this activation is a voltage-gated Ca^{2+} influx [31,32]. However, more recent studies have shown that excess K^+ activates Ca^{2+} waves similar to those at fertilization [29], so it is not unlikely that the activation mechanisms in K^+-activated eggs involve $InsP_3$ synthesis like those in fertilized eggs. In support of this hypothesis, we report that U-73122 also prevented activation by excess K^+. Finally, we show that *Chaetopterus* sperm also contain a factor with similar enzymatic properties to those of mammalian sperm factor. This suggests that such factors may be widespread in the animal kingdom.

Methods

Gamete preparation. Adult specimens of *Chaetopterus pergamentaceus* were obtained either from the Marine Resources Division, Marine Biological Laboratory, Woods Hole, MA or from Cape Fear Biological, Southport, NC. Gametes were obtained, handled, fertilized or artificially activated with 50 mM excess KCl as described previously [33]. U-73122 and U-73343 were obtained from Calbiochem and used from 5 mM stock solutions in DMSO. Other reagents were obtained from Sigma–Aldrich.

Chaetopterus egg homogenate. Vitelline envelope-free metaphase I arrested oocytes were washed in calcium-free artificial seawater (CaFASW), then homogenized in GluIM+(GluIM [333 mM *N*-methylglucamine, 333 mM potassium acetate, 27 mM Hepes, and 1.3 mM magnesium chloride, pH 7.2], protease inhibitors [100 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, and 25 μg/ml aprotinin], and an ATP regenerating system [10 mM ATP, 100 mM phosphocreatine, and 0.1 U/ml creatine phosphokinase]), and centrifuged 14,000*g* for 10 s in a microfuge. The supernatants were aliquotted and stored at −80 °C until use.

Sperm extracts. Boar sperm extract, obtained as described [17], was the generous gift of Dr. Karl Swann, University College, London, UK. It was dissolved at 50 mg/ml in water.

Chaetopterus sperm extract was prepared by a similar method. Sperm were separated from gonadal debris by differential centrifugation [33]. Then the sperm were washed in CaFASW and centrifuged 10,000 rpm for 5 min at 4 °C. The pellet was suspended in an equal volume of extraction buffer (120 mM KCl, 20 mM Hepes, pH 7.5, 100 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). The sperm were lysed by five cycles of freeze-thawing. Lysates were centrifuged 100,000*g* for 1 h at 4 °C. Supernatants were collected and concentrated using Centricon C-30 filters (Amicon). Concentrated extracts (~10 mg/ml) were frozen at −80 °C in 5–10 μl aliquots.

Calcium release in vitro. Homogenates were slowly diluted 1:20 in Mannitol IM+ ([250 mM mannitol, 250 mM potassium gluconate, 20 mM Hepes, and 1 mM $MgCl_2$, pH 7.2], protease inhibitors [100 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, and 25 μg/ml aprotinin], and an ATP regenerating system [10 mM ATP, 100 mM phosphocreatine, and 0.1 U/ml creatine phosphokinase]) over 3 h at 17 °C. Fluo-3 was added to a final 3 μM.

$InsP_3$ was added to a final 2 μM; sperm extracts were added to a final 20–100 μg/ml. In some experiments, 1 μl of of 5 mg/ml *Chaetopterus* sperm extract and 1 μl of 5 mM phosphatidylinositol bisphosphate (PIP_2) were mixed, incubated 5 min at room temperature, and added to a final sperm factor concentration of 20–100 μg/ml. The inhibitors (U-73122 and U-73343) were added at a final concentration of 10 μM. Changes in free Ca^{2+} were measured on either a Deltascan (Photon Technology International, Monmouth Junction, NJ) or an LS55 (Perkin–Elmer, Boston, MA) as described [30].

Results and discussion

Boar sperm factor added to *Chaetopterus* egg homogenates elicited a release of Ca^{2+} after a delay (Fig. 1A). This response is similar to that of sea urchin egg homogenates [27,34]. Similarly, sperm factor prepared from *Chaetopterus* also elicited a Ca^{2+} release after a delay (Fig. 1B).

Having established that sperm factor can elicit Ca^{2+} release in *Chaetopterus* egg homogenates, we tested by homologous desensitization whether or not it releases Ca^{2+} through $InsP_3$-gated channels. Prior addition of saturating amounts of $InsP_3$ to the homogenates abolished their ability to release Ca^{2+} in response to sperm factor (Fig. 2A). Furthermore, prior exposure to sperm factor from *Chaetopterus* (Fig. 1B) or boar (data not shown) greatly reduced the response to $InsP_3$. Together, these results demonstrate that sperm factor releases Ca^{2+} through $InsP_3$-sensitive stores. To test whether *Chaetopterus* sperm factor contains a PLC, sperm factor was incubated with exogenous PIP_2 and added to the egg homogenate. The release was immediate as observed upon $InsP_3$ addition (Fig. 2B). When PIP_2 and sperm factor were simultaneously added to the egg homoge-

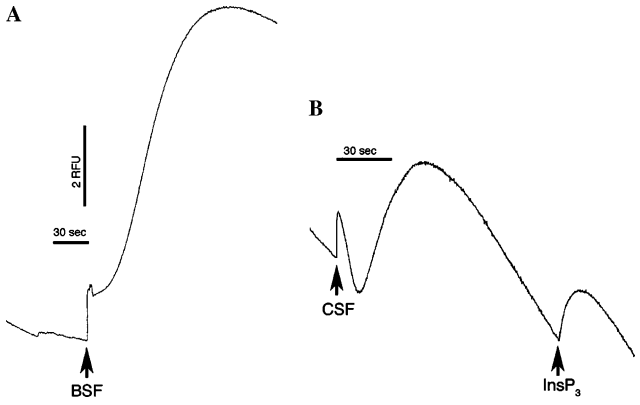

Fig. 1. Sperm factor from either mammalian or homologous sources elicited Ca^{2+} release in *Chaetopterus* egg homogenates. (A) Heterologous boar sperm factor (BSF) was added at the indicated time and fluorescence was monitored. (B) Homologous *Chaetopterus* sperm factor (CSF) prepared similarly to the boar sperm factor was added at the indicated time and fluorescence was monitored. Prior exposure to homologous sperm factor substantially reduced the response to $InsP_3$. The horizontal scale bar represents 30 s; the vertical bar represents 2 RFU. In the other experiments, RFU values are not given, as all figures have been drawn to the same vertical scale.

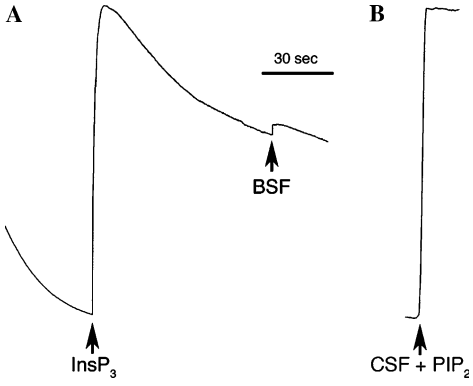

Fig. 2. Involvement of $InsP_3$ in *Chaetopterus* egg homogenate Ca^{2+} release in response to sperm factor. (A) $InsP_3$ was added at the indicated time and fluorescence was monitored. After the released Ca^{2+} was resequestered, BSF was added. There was no release, demonstrating that the Ca^{2+} releasing activity of sperm factor requires $InsP_3$. (B) *Chaetopterus* sperm factor was preincubated for 5 min with PIP_2 and added to egg homogenate. Ca^{2+} was immediately released as upon addition of $InsP_3$ indicating that the sperm factor contains a PLC activity that synthesizes $InsP_3$.

nate, Ca^{2+} release occurred after a delay as observed with sperm factor alone (data not shown). This establishes that *Chaetopterus* sperm contain a PLC activity with similar activity to that of mammalian sperm factor and is the first demonstration of such an activity in an invertebrate sperm.

To test whether PLC activity is required at fertilization, we tested the effects on fertilization of U-73122, a competitive inhibitor of agonist-induced PLC activation of Ca^{2+} release [35], and that of U-73343, an analog of U-73122 that is a very weak inhibitor of PLC. U-73122 inhibited egg activation in a concentration-dependent manner (Fig. 3A), indicating that egg activation is PLC-dependent. In addition, the compound also inhibited activation by excess K^+ (Fig. 3A). By contrast, U-73343, a much weaker PLC antagonist, was ineffective at inhibiting activation by either sperm or excess K^+ (Fig. 3B).

If sperm factor-induced Ca^{2+} release is PLC-dependent, U-73122 should block it but not $InsP_3$-induced Ca^{2+} release. Prior addition of 10 μM U-73122 to the homogenate inhibited release by the sperm factor (Fig. 4A), but not by $InsP_3$ (Fig. 4B). This demonstrates that U-73122 does not prevent sperm factor-induced Ca^{2+} release by interfering with $InsP_3$ binding or response, directly. Rather, it must block Ca^{2+} release by interfering with $InsP_3$ synthesis. By contrast, similar concentrations of U-73343 did not inhibit Ca^{2+} release from the homogenate either by sperm factor or $InsP_3$ (Figs. 4C and D).

Sperm extracts microinjected into mammalian or nemertean eggs elicit Ca^{2+} oscillations after a delay [13,17,18,36]. When introduced into sea urchin homogenates, they elicit a single Ca^{2+} release event after a delay [17,34]. The fact that *Chaetopterus* egg microsomes do not undergo Ca^{2+} oscillations in response to sperm factor, even though the eggs undergo oscillations at fertilization, demonstrates that the oscillatory behavior is a property of intact eggs that is lost upon homogenization and in vitro assay.

These delays have been interpreted to indicate the synthesis of some kind of second messenger(s). Because sea urchin egg homogenates release Ca^{2+} in response to multiple second messengers, it could not be unambiguously established that the release was due to any particular second messenger.

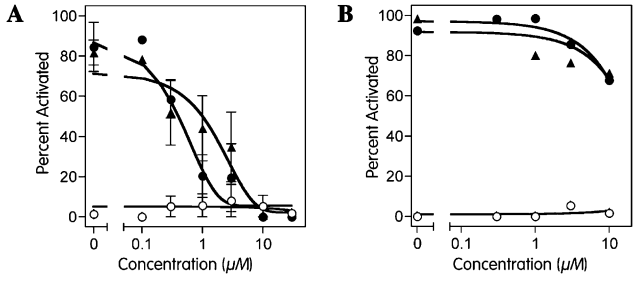

Fig. 3. Effects of strong and ineffective PLC inhibitors on egg activation in *Chaetopterus*. (A) The indicated concentrations of U-73122 were added to unfertilized eggs. After 15 min, sperm or 50 mM excess K^+ was added. After 30 min, eggs were scored for activation as evidenced by polar body formation. (B) The indicated concentrations of U-73343 were added to unfertilized eggs. After 15 min, sperm or excess K^+ was added. After 30 min, eggs were scored for activation as evidenced by polar body formation. Each point represents the means ± SD of 3–5 separate experiments. Symbols: filled circles—fertilized eggs; triangles—KCl-activated eggs; and open circles—unfertilized eggs that received no further treatment.

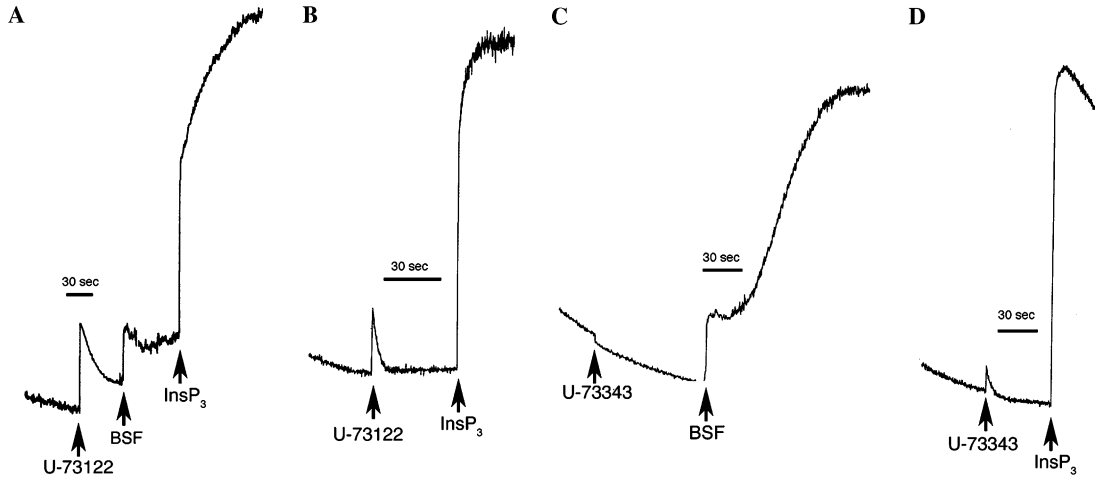

Fig. 4. Effects of strong and ineffective PLC inhibitors on Ca^{2+} release from *Chaetopterus* egg homogenates in response to $InsP_3$ and sperm factor. (A) At the indicated times, the potent PLC inhibitor U-73122 and BSF were added to the homogenate and fluorescence was monitored. After U-73122 addition, BSF failed to elicit Ca^{2+} release. (B) At the indicated times, U-73122 and $InsP_3$ were added to the homogenate and fluorescence was monitored. U-73122 had no effect on $InsP_3$-induced Ca^{2+} release. (C) At the indicated times, the weak PLC inhibitor U-73343 and BSF were added to the homogenate and fluorescence was monitored. U-73343 had no effect on BSF-induced Ca^{2+} release. (D) At the indicated times, U-73343 and $InsP_3$ were added to the homogenate and fluorescence was monitored. U-73343 had no effect on $InsP_3$-induced Ca^{2+} release.

By contrast, studies of mouse eggs in vivo suggested that PLC activity was required for sperm factors to elicit Ca^{2+} oscillations in living eggs. These studies led to the identification of a novel PLC isoform, PLCζ, that is sperm-specific and able to elicit Ca^{2+} oscillations in living eggs and Ca^{2+} release from sea urchin homogenates [13,28].

The fact that an agent can elicit such Ca^{2+} release does not necessarily mean that it is the agent active at fertilization. In fact, experiments with sea urchin homogenates failed to show a clear involvement of PLC or $InsP_3$ in Ca^{2+} release in response to sperm factor [27,34]. Our experiments were designed to test this directly. Since *Chaetopterus* egg homogenates, like mammalian eggs, do not respond to second messengers other than $InsP_3$ [30], we tested whether PLC activity is necessary for mammalian sperm factor to release Ca^{2+} from them. The results confirmed that PLC activity is present in the sperm extracts and showed that it is essential for the functioning of crude sperm extracts in vitro. These results demonstrate that PLC activity from sperm is both necessary and sufficient to elicit Ca^{2+} release in and activate eggs.

The structure of PLCζ suggested that it might be widespread in animal sperm. Indeed, crude sperm factors have been prepared from nemertean worms [18], although their biochemical properties have not been reported. Our earlier, preliminary results on *Chaetopterus* sperm failed to demonstrate a factor that could release Ca^{2+} from egg homogenates [30], but the present study clearly shows that it exists. Apparently, the difference is due to improved methods of sperm factor preparation and differences in the assay conditions. In this study, sperm factor activity was assayed in Mannitol IM instead of GluIM, and protein concentrations of the sperm lysates were at least an order of magnitude higher than in our previous studies in which the lysates were not concentrated. Thus, our previous assays were not sensitive enough to detect the activity.

Our finding that U-73122 inhibited parthenogenetic activation by excess K^+ at similar concentrations to those that blocked activation by sperm may seem surprising, as K^+ has long been supposed to act by depolarizing the plasma membrane and allowing a Ca^{2+} influx. However, K^+-induced egg activation is also associated with repetitive Ca^{2+} transients that are similar to those in response to fertilization [29]. Since Ca^{2+} oscillations in eggs are associated with $InsP_3$ synthesis [11,12,37–39], it should not be surprising that K^+-activation also involves PLC activity. This result also indicates that the eggs contain a PLC that can contribute to the activation process. The Ca^{2+} influx in response to excess K^+ might thus activate the egg by activating a Ca^{2+}-dependent PLC present in the egg, whereas sperm activate the egg through a Ca^{2+}-dependent PLC of their own.

Acknowledgments

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