A mammalian sperm cytosolic phospholipase C activity generates inositol trisphosphate and causes Ca^{2+} release in sea urchin egg homogenates

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Abstract Injection of sperm extracts triggers Ca^{2+} oscillations in mammalian eggs similar to those seen at fertilisation. Here, we show that addition of sperm extracts to sea urchin egg homogenates causes Ca^{2+} release and inositol 1,4,5-trisphosphate ($InsP_3$) production. Furthermore depleting homogenates of phosphatidylinositol lipids using a phosphatidylinositol-specific phospholipase C blocked the sperm extract from causing $InsP_3$ production and a Ca^{2+} rise. A response could be recovered by the addition of phosphatidylinositol 4,5-bisphosphate to either sperm extracts or egg homogenates. These data indicate that sperm extracts contain an $InsP_3$-generating phospholipase C which may play a role in Ca^{2+} release at fertilisation.

Key words: Egg; Sperm; Calcium; Inositol trisphosphate; Phospholipase C

1. Introduction

A rise in cytosolic Ca^{2+} levels is the essential signal for activation of the egg at fertilisation in all species studied [1,2]. In most species this increase involves Ca^{2+} release from internal stores but the mechanism by which the sperm triggers Ca^{2+} release is unresolved. One proposal is that the sperm introduces a soluble factor which triggers Ca^{2+} release following gamete membrane fusion [3–6]. The main support for this mechanism is based on the finding that cytosolic extracts prepared from sperm cause Ca^{2+} increases when microinjected into eggs. The factor in the extracts is not species specific and in mammals injecting extracts made from the sperm of hamster, human and pig can cause Ca^{2+} oscillations similar to those observed at fertilisation in hamster, human or mouse eggs [2,6]. Injection of cytosolic sperm extracts have also been shown to trigger Ca^{2+} oscillations in the oocytes of Nemertean worms [5]. The sperm factor that triggers Ca^{2+} oscillations is of high molecular weight and both trypsin-sensitive and heat-labile, suggesting it is a protein [3,5]. The exact nature of the sperm factor remains unresolved since many sperm factor candidates have been proposed but no recombinant protein has yet been found to cause Ca^{2+} oscillations similar to those seen at fertilisation [4,7].

Independent of whether Ca^{2+} release occurs via soluble sperm factors or plasma membrane receptors there are a number of mechanisms that may operate to trigger Ca^{2+} release at fertilisation. The strongest evidence involves the generation of inositol trisphosphate ($InsP_3$) since $InsP_3$ microinjection causes Ca^{2+} oscillations [2,8] and sperm-induced Ca^{2+} release is blocked by the $InsP_3$ receptor ($InsP_3R$) antagonist heparin [9–11] and by a functionally inhibitory monoclonal antibody raised against the $InsP_3R$ [12]. The $InsP_3$ generated at fertilisation may be produced by the action of a phosphatidylinositol(4,5)bisphosphate-specific phospholipase C (PIP_2-PLC) within the egg since pre-incubation of mouse eggs with U73122, a PIP_2-PLC inhibitor, blocks sperm-induced Ca^{2+} oscillations [13]. Measurement of PI lipid turnover has shown that it increases in eggs at fertilisation in the sea urchin [14] and frog [1,15]. No study has reported PI changes in mammalian eggs at fertilisation.

This study has examined the mechanism by which the mammalian sperm factor causes Ca^{2+} release using egg homogenates prepared from the sea urchin *Lytechinus pictus*. The sea urchin egg homogenate assay system has the advantage that it has been extensively characterised and shows Ca^{2+} release in response to the addition of three different Ca^{2+}-releasing agents: $InsP_3$, cyclic ADPRibose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [16]. Boar sperm extracts were previously shown to trigger Ca^{2+} release from the sea urchin egg homogenates [17]. This result is not surprising given both sea urchin eggs [18,19] and mammalian eggs [20] contain both $InsP_3Rs$ and ryanodine receptors (RyRs). Our preliminary data had suggested that the sperm factor acts via both the $InsP_3R$ and the RyR [17]. However, we now demonstrate that it causes release of Ca^{2+} only through the $InsP_3$ pathway.

2. Materials and methods

All chemicals were from Sigma unless stated otherwise. PIP_2 (5 mM) was dissolved in distilled water. A *Bacillus cereus* PI-PLC [21] was from Sigma (P5542).

Boar sperm cytosolic extracts were prepared by a modification of previously described procedures [3]. Boar semen was washed at $800\times g$ for 10 min in PBS (Ca^{2+}-free), pH 7.5. In the final wash 200 μM PMSF and 2 μg/ml leupeptin were added. Excess saline was removed and the pellet lysed by cycles of freeze-thawing in liquid N_2. Lysates were centrifuged at $100\,000\times g$ for 1 h at 4°C. The supernatant was then concentrated, rediluted and reconcentrated into 120 mM KCl, 20 mM HEPES, pH 7.5 with 200 μM PMSF on Centricon C-30 cut-off filters (Amicon, Gloucestershire, UK). They were aliquotted, frozen in liquid N_2 and stored at −80°C. The protein concentration of the sperm extracts used for assay in the homogenate varied between 10–30 mg/ml, a range similar to that reported to be effective previously [17]. Some sperm extracts underwent extensive cycles of freeze-thawing in liquid N_2 and a water bath of 50°C to inactivate them. Such extracts had no Ca^{2+}-releasing ability.

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Abbreviations: bsf, boar sperm factor; cADPR, cyclic ADPRibose; IM, intracellular medium; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; NAADP, nicotinic acid adenine dinucleotide phosphate; PI, phosphatidylinositol; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PLC, phospholipase C; PI-PLC, PI-specific PLC; PIP_2-PLC, PIP_2 specific PLC; RyR, ryanodine receptor

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Homogenates (2.5%) of unfertilised *Lytechinus pictus* eggs (Marinus, Long Beach, CA, USA) were prepared as described previously [22] using 250 mM potassium gluconate; 250 mM mannitol; 20 mM HEPES pH 7.2; 1 mM $MgCl_2$; 1 mM ATP; 10 mM phosphocreatine; 10 IU/ml creatine phosphokinase; 1 μg/ml oligomycin; 1 mM sodium azide; 3 mM fluo-3. All IM was treated with iminidiacetic acid chelating resin (1% v/v) to remove heavy metal contamination before the addition of 1 mM $MgCl_2$. Fluorimetry was performed at 17°C using 500 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter.

For $InsP_3$ mass assay measurements various additions were made to 1-ml aliquots of 2.5% sea urchin egg homogenates before freezing in liquid N_2 and storage at −30°C. Protein was extracted by incubation with 0.2 volumes 20% perchloric acid for 20 min. Protein was sedimented by centrifugation at 2000×*g* for 15 min. The supernatant was neutralised to pH 7.4–7.5 with ice-cold 1.5 M KOH, 60 mM HEPES, then centrifuged at 2000×*g* for 15 min. All conditions were on ice and centrifugations were at 4°C. The supernatant, later assayed for $InsP_3$, was stored at −80°C. The $InsP_3$ content of the samples was measured using a commercial $InsP_3$ assay kit (TRK1000; Amersham, UK) according to the manufacturer's instructions. Briefly samples were diluted in 0.1 M Tris, 4 mM EDTA, 4 mg/ml BSA, trace ^{3}H-$InsP_3$ and microsomes. The mix was incubated on ice for 15 min, then centrifuged at 2000×*g* for 10 min. The pellet was resuspended in water and incubated at 25°C for 10 min. The mixture was counted in triplicate in 10 ml Optiphase (Wallac, UK) scintillant on a Hewlett Packard counter.

3. Results and discussion

Boar sperm extract when added to sea urchin egg homogenate caused a rapid rise in Ca^{2+} (Fig. 1a). This response was characterised further by utilising the phenomenon of homologous desensitisation which occurs in sea urchin egg homogenates; additions of maximal amounts of either $InsP_3$, cADPR or NAADP cause a desensitisation to subsequent additions of the same agent but not to the other two. It was found that homogenates which were desensitised to the cADPR receptor and the NAADP receptor still respond to additions of the boar sperm extract (Fig. 1b). However, desensitisation of the $InsP_3R$ by repeated application of maximal doses of $InsP_3$ abolished the response to sperm extract completely, but as expected this left the response to cADPR (Fig. 1c) and NAADP (not shown) unaffected. The addition of heparin, a well known $InsP_3$ competitive inhibitor, completely blocked the action of the sperm extract at 100 μg/ml concentrations (Fig. 1d) consistent with sperm extract action through the $InsP_3R$.

The above data imply the production of $InsP_3$ by the sperm extract when added to homogenate. Therefore homogenates were treated with various agents before analysis of $InsP_3$ production by an $InsP_3$ mass assay. In control experiments $InsP_3$ was not detected in either the prepared sperm extracts or in the homogenate to which no additions were made (Table 1). However, following addition of 10 μl sperm extract (10–30 mg/ml protein) to 1 ml of homogenate $InsP_3$ production was constantly found (Table 1) confirming the desensitisation experiments.

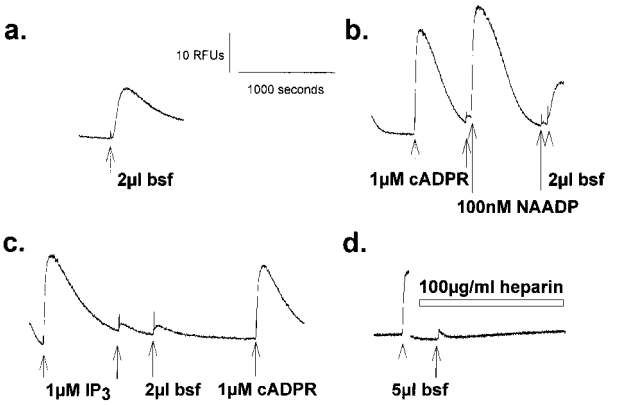

Fig. 1. The boar sperm factor released Ca^{2+} through the $InsP_3R$. a: 2 μl bsf (10–30 mg/ml protein) when added to untreated homogenate caused a large rise in Ca^{2+}; b: 2 μl bsf (10–30 mg/ml protein) when added to sea urchin egg homogenates that had been desensitised to both cADPR and NAADP induced a rise in Ca^{2+}. Note the response to bsf only appears small because of the relatively large Ca^{2+} release generated by maximal doses of the other two agents. In fact the change in relative fluorescence units (RFU) is 13, similar to that in a. c: 2 μl bsf (10–30 mg/ml protein) added to egg homogenate desensitised to $InsP_3$, by additions of 1 μM $InsP_3$, did not release Ca^{2+} although homogenates responded to subsequent 1 μM cADPR addition. Ca^{2+} release in cADPR and NAADP desensitised homogenate was observed for boar sperm extract in 20 other experiments. Similarly 20 other experiments showed no Ca^{2+} release with sperm extract following $InsP_3$ desensitization. d: 5 μl bsf (10–30 mg/ml protein) gave maximal Ca^{2+} release when added to homogenate but its action was completely ablated by treating homogenate with 100 μg/ml heparin.

In further experiments it was found that the production of $InsP_3$ required endogenous PI lipids since incubation of sea urchin egg homogenate with 100 μg/ml of bacterial PI-PLC for periods longer than 12 h resulted in a homogenate that showed no $InsP_3$ production in response to sperm extract addition (<7 pmol/ml, $n=2$). The activity of the bacterial PI-PLC, which has been reported to specifically hydrolyse PI over other phospholipids [21], was therefore investigated further in homogenates. Egg homogenates treated with 33–100 μg/ml bacterial PI-PLC for more than 12 h gave no Ca^{2+} response to sperm extract (Fig. 2a, $n=4$) consistent with the results of the $InsP_3$ mass assay measurements. Such PI-PLC treated homogenates were still functional for other kinds of response since they maintained a low extraluminal Ca^{2+} level, they responded with a Ca^{2+} rise after $InsP_3$ addition (Fig. 2a), and they generated a cADPR mediated Ca^{2+}

Table 1
$InsP_3$ production in sea urchin egg homogenate following the addition of various agents

Addition	Added to 1 ml of	Measured $InsP_3$ (pmol)
10 μl bsf	Buffer	<7
No additions	Egg homogenate	<7
10 μl bsf	Egg homogenate	202 ± 45
10 μl bsf, heat-treated[a]	Egg homogenate	<7
10 nmol Ca^{2+}	Egg homogenate	<7

$InsP_3$ was measured using a commercial $InsP_3$ mass assay, as described in Section 2. Levels of $InsP_3$ are reported for 1 ml of homogenate. Results are expressed as mean ± standard error of the mean from 6 separate experiments. The detection limit of the assay was 7 pmol/ml $InsP_3$.
[a]Heat-treated bsf was obtained as described in Section 2.

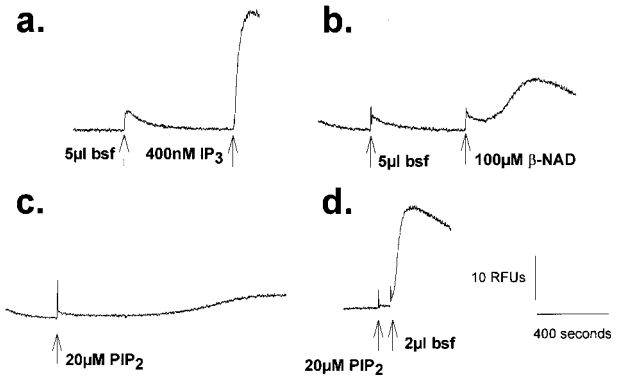

Fig. 2. The effect of treating sea urchin egg homogenates with a *Bacillus cereus* PI-PLC. a: The response to 5 µl bsf (10–30 mg/ml), but not 400 nM $InsP_3$, was abolished by depletion of PI lipids in the homogenate by overnight incubation with 100 µg/ml PI-PLC. b: PI-PLC treated homogenates showed a normal NAD cyclase activity, as observed by the generation of cADPR following addition of 100 µM β-NAD. c: The addition of 20 µM PIP_2 to homogenates that contained PI-PLC caused a very small rise in Ca^{2+} over the 25 min of recording. d: When PIP_2 was added to PI-PLC-treated homogenates the bsf response was recovered, judged by the large rise in Ca^{2+} following its addition.

rise after NAD addition (Fig. 2b, $n=2$). These findings suggest that the inhibition of the sperm extract response by PI-PLC was specific, non-toxic and due to depletion in the homogenates of PI lipid rather than a non-specific effect. To further investigate this effect of PI lipid depletion on the response to sperm extracts PIP_2 was added back to the homogenate. When 20 µM PIP_2 was added to such PI-depleted, PI-PLC containing homogenate a small rise in Ca^{2+} was observed with a distinct latency of several minutes (Fig. 2c). This was probably due to the ability of the bacterial PI-PLC to hydrolyse PIP_2 in addition to PI, since it was not observed in homogenates without added PI-PLC (not shown). In PI-PLC containing homogenates to which PIP_2 was added the addition of sperm extract caused an immediate large rise in Ca^{2+} (Fig. 2d, $n=3$). Therefore PIP_2 restored the ability of PI-depleted homogenates to respond to the sperm extract. These data suggest the involvement of a PIP_2-PLC and PIP_2 in the sperm factor response.

Sperm extract may contain a relevant PIP_2-PLC introduced into the egg or alternatively may activate a PIP_2-PLC within the egg. To examine this sperm extracts were diluted until no Ca^{2+} rise was observed over an extended time period. Typically this was a 1:50 dilution of a 10–30-mg/ml extract (Fig. 3). The addition of 20 µM PIP_2 to the homogenate resulted in an improved sperm extract response, presumably due to an increased concentration of substrate in the assay system. When 1 µl of the 1:50 10–30-mg/ml sperm extract was incubated for 5 min with 1 µl 20 µM PIP_2 and then added to homogenates an immediate and large rise in Ca^{2+} was seen (Fig. 3). This indicates that the PIP_2-PLC activity which generates $InsP_3$ from PIP_2 resides within the sperm extract itself.

The present observations on the mechanism of action of sperm extract in sea urchin egg homogenates clearly suggest that it involves the production of $InsP_3$. Not only was $InsP_3$ detected by a mass assay in response to sperm extract addition, but a bacterial PI-PLC, that was used to deplete endogenous PI lipids, specifically abolished the ability of sperm extracts to cause $InsP_3$ production and Ca^{2+} release. Despite the fact that a Ca^{2+}-activated PIP_2 breakdown has been demonstrated in isolated sea urchin egg cortices [23,24], the sea urchin egg homogenate does not present any evidence for an endogenous Ca^{2+}-dependent $InsP_3$ production. The lack of Ca^{2+}-activated $InsP_3$ production within the homogenate is in accord with the phenomenon of homologous desensitisation. Large Ca^{2+} rises are seen when homogenates are desensitised to cADPR and NAADP, and yet this does not affect their ability to respond to additions of $InsP_3$ [22]. Therefore, these data suggest that the sperm extract contains a factor which specifically activates the production of $InsP_3$ and that this is the cause rather than an effect of the Ca^{2+} release.

In collaboration with others we previously reported that sperm extracts caused Ca^{2+} release in sea urchin egg homogenate [17]. There are, however, three main differences between the present and past findings. Firstly, here we report that sperm extract releases Ca^{2+} only through the $InsP_3R$, with no involvement of the RyR. Desensitisation of the $InsP_3R$ abolished the sperm extract response whereas previously it was reported under similar conditions to still cause Ca^{2+} release. Secondly, here we find that a sperm PLC, acting on egg PIP_2 to yield the low molecular weight messenger $InsP_3$, is responsible for Ca^{2+} release. Previously a sperm protein-egg protein interaction to yield a high molecular weight novel Ca^{2+}-releasing agent was proposed. Thirdly, consistent with the present observations, we find that heparin, an $InsP_3$ antagonist, ablates sperm extract action. Previously it was found necessary to combine heparin with 8-NH_2-cADPR, a cADPR antagonist to block a sperm extract response. We feel that differences in sperm extract batches or preparations of egg homogenate cannot account for these contrasting results since in our laboratory the present data have been obtained with

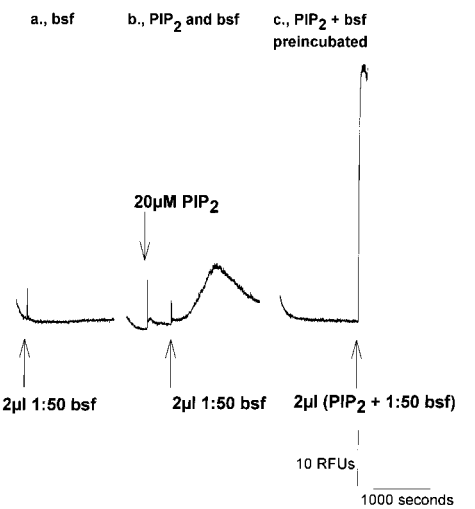

Fig. 3. PIP_2-PLC activity resides in the boar sperm extract. a: Very diluted bsf (2 µl of a 1:50 dilution of a 10–30-mg/ml extract) gave no rise in Ca^{2+}. b: When 20 µM PIP_2 was added to the homogenate the presumed substrate concentration was effectively increased so that the added bsf now caused a rise in Ca^{2+} with a small delay. c: When 1 µl 1:50 bsf was incubated with 1 µl 5 mM PIP_2 for 5 min at room temperature and then added to the homogenate (final PIP_2 concentration in homogenate of 10 µM) the preincubated bsf caused a very large rise in Ca^{2+}. The same result was obtained using different batches of diluted sperm extracts in four separate experiments.

more than 20 different batches of sperm extract and 3 different preparations of sea urchin egg homogenate. We have not been able to reconcile the two sets of contrasting results which were carried out in different laboratories. In our homogenates the large volume additions made previously (20–30 μl) usually gave spurious Ca^{2+} rises. In the present study only small volume additions (2–5 μl) were ever made. We conclude that, if factors in sperm extract do cause any Ca^{2+} release via the RyR, then it has very little physiological significance compared to the $InsP_3R$.

Previous studies have shown that sperm contains PIP_2 specific PLCs [25–27]. These PLCs have been implicated in the acrosome reaction of the sperm, although some workers have implied a potential role of PIP_2-PLC in egg activation at fertilisation [25]. Since a sperm PIP_2-PLC appears responsible for Ca^{2+} release in homogenates, it will be important to establish whether precisely the same protein in the extracts that mediates this form of Ca^{2+} release also triggers the Ca^{2+} oscillations in intact eggs. Full purification of the factors has not yet been achieved, but in each system the protein(s) involved are sperm specific and of high molecular weight [3,17]. If the factor active in homogenates proves to be the same as that active in intact eggs then our data may link two previously opposing theories of signal transduction at fertilisation. The sperm factor, a PIP_2-PLC, introduced after fusion generates $InsP_3$ that in other somatic systems is normally linked to receptor-stimulated signalling pathway.

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