Different Ca^{2+} -releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin egg homogenate and mouse eggs

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A soluble phospholipase C (PLC) from boar sperm generates InsP_3 and hence causes Ca^{2+} release when added to sea urchin egg homogenate. This PLC activity is associated with the ability of sperm extracts to cause Ca^{2+} oscillations in mammalian eggs following fractionation. A sperm PLC may, therefore, be responsible for causing the observed Ca^{2+} oscillations at fertilization. In the present study we have further characterized this boar sperm PLC activity using sea urchin egg homogenate. Consistent with a sperm PLC acting on egg $\text{PtdIns}(4,5)\text{P}_2$, the ability of sperm extracts to release Ca^{2+} was blocked by pre-incubation with the PLC inhibitor U73122 or by the addition of neomycin to the homogenate. The Ca^{2+} -releasing activity was

also detectable in sperm from other species and in whole testis extracts. However, activity was not observed in extracts from other tissues. Moreover recombinant $\text{PLC}\beta 1$, $-\gamma 1$, $-\gamma 2$, $-\delta 1$, all of which had higher specific activities than boar sperm extracts, were not able to release Ca^{2+} in the sea urchin egg homogenate. In addition these PLCs were not able to cause Ca^{2+} oscillations following microinjection into mouse eggs. These results imply that the sperm PLC possesses distinct properties that allow it to hydrolyse $\text{PtdIns}(4,5)\text{P}_2$ in eggs.

Key words: fertilization, Ca^{2+} oscillations, inositol 1,4,5-trisphosphate.

INTRODUCTION

At fertilization, in all species studied, the sperm causes egg activation by increasing intracellular Ca^{2+} [1,2]. This increase in egg Ca^{2+} may take the form of a single rise, as in sea urchins [3] and frogs [4], or else a series of oscillations, as in mammals [5], ascidians [6] and nemertean worms [7]. There have been three different models to account for how the sperm causes Ca^{2+} release in the egg at fertilization [8]. The first is that the sperm acts as a conduit for Ca^{2+} entry at the site of fusion. This model is consistent with the finding that sperm–egg membrane fusion occurs before Ca^{2+} release in sea urchin [9] and mouse eggs [10,11]. However, direct local measurement of Ca^{2+} at the time of fusion in the mouse has found no evidence of Ca^{2+} influx from the fertilizing sperm [11]. The other two models are that the sperm interacts with an egg receptor to cause Ca^{2+} release [12], or that a soluble sperm factor is released following gamete fusion [13].

In the receptor model the egg receptor is either coupled to a G protein, to activate phospholipase $C\beta$ ($\text{PLC}\beta$), or to a tyrosine kinase to activate $\text{PLC}\gamma$. However, in starfish it has been suggested that at fertilization both $\text{PLC}\beta$ and $\text{PLC}\gamma$ may be activated [14]. Therefore the activation of one or more of these PLC isoforms may lead to the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to generate InsP_3 within the egg [12]. The receptor model is supported indirectly by the finding in frog [15] and sea urchin eggs [16,17] that an increase in InsP_3 production occurs at fertilization. Furthermore, the PLC enzyme inhibitor U73122 has also been shown to inhibit Ca^{2+} release at fertilization in both sea urchin [17] and mouse eggs [18]. Neomycin, another inhibitor of PLC, which acts by binding to $\text{PtdIns}(4,5)\text{P}_2$ [19], can also

block Ca^{2+} release in fertilizing sea urchin eggs [20]. However, the specificity of these drugs and their site of inhibition remains to be clarified, since U73122 can affect cyclic-GMP-induced–cyclic ADP-ribose (cADPR) production [21], and neomycin also inhibits ryanodine-sensitive Ca^{2+} release in muscle tissues [22]. In addition, despite the observed changes in InsP_3 in fertilizing eggs, no egg receptor has been found which when activated causes the characteristic Ca^{2+} changes observed in eggs at fertilization [12]. The mechanism by which the sperm stimulates InsP_3 production has therefore remained unclear.

The sperm factor hypothesis is an alternative to the receptor-mediated mechanism [13,23]. It is based on the finding that the microinjection into eggs of cytosolic sperm extracts [7,24–26], but not other tissue extracts [7,24,26], triggers Ca^{2+} oscillations identical to those observed at fertilization. Such Ca^{2+} oscillation-inducing activity has been reported in sperm extracts from mammals [24], nemertean worms [7] and ascidians [25] when injected into eggs of the homologous species. In mammals the source of sperm extract need not be the same species as the egg, since human [27], boar [26,28,29] and hamster sperm extracts [28,30,31] are effective in heterologous eggs. These findings suggest that in mammals at least, the factor is functionally conserved. The sperm factor has also been shown to be both heat- and trypsin-sensitive, and therefore proteinaceous in nature [7,23,24].

The identification of the sperm factor protein is unresolved. Although it is generally agreed that the molecular mass of the factor is greater than 30 kDa, some suggest a molecular mass between 30 and 100 kDa [25,32], while others suggest >100 kDa [24]. An initial candidate sperm factor, a 33 kDa deaminase, was correlated with the active Ca^{2+} -releasing fractions when hamster

Abbreviations used: cADPR, cyclic ADP-ribose; NAADP, nicotinic acid–adenine dinucleotide phosphate; PLC, phospholipase C.

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sperm extract was chromatographically separated [33]. However, the Ca^{2+} -releasing sperm factor must have been another protein in the active fractions since recombinant deaminase has no Ca^{2+} -releasing ability [34] and sperm extracts lacking deaminase activity are still able to cause Ca^{2+} oscillations in mammalian eggs [29,35]. Another candidate is the sperm-specific truncated version of the c-kit receptor, referred to as tr-kit [36,37]. Unfortunately its potential role at fertilization cannot yet be verified, since the tr-kit protein has not been shown to cause Ca^{2+} oscillations in any egg.

We have shown recently that boar sperm cytosolic extracts trigger Ca^{2+} release in sea urchin egg homogenates [38]. Such egg homogenates have been used by a number of groups to study Ca^{2+} homeostasis and to establish the ability of novel pyridine nucleotide metabolites to trigger Ca^{2+} release [39,40]. Using the sea urchin egg homogenate we found that the sperm extract-induced Ca^{2+} release was caused by the action of a sperm-derived PLC, which generated InsP_3 within the homogenate [38]. The same sperm PLC activity also appears to be responsible for causing Ca^{2+} oscillations in intact mouse eggs [35]. It is therefore our proposal that the sperm factor is a PLC. This may help unite some aspects of the receptor and sperm factor theories of signalling at fertilization. However, some of the basic characteristics of the sperm PLC are not resolved. In the current study we have used the sea urchin egg homogenate to investigate the nature and potential role of the putative sperm PLC in Ca^{2+} release in eggs. Our findings suggest that the mammalian sperm factor PLC is either sperm specific, or else sperm-specifically regulated, since some common isoforms of PLC by themselves are unable to initiate Ca^{2+} release in eggs. In addition, we suggest that some of the previously reported inhibitory effects of PLC antagonists in eggs are due to an action upon a sperm-derived PLC, rather than upon PLCs present in the egg.

EXPERIMENTAL

All chemicals were from Sigma unless stated otherwise. U73122 (Calbiochem) was dissolved in chloroform (50 mM) or DMSO (5 mM).

Preparation of tissue extracts

Boar sperm extracts were prepared as described previously [38]. Briefly semen was washed in Ca^{2+} -free PBS, with the addition of protease inhibitors in the final wash. The sperm pellet was then lysed by freeze thawing in liquid nitrogen. Lysates were centrifuged at 100 000 g for 1 h at 4 °C. The supernatant was concentrated, rediluted and reconcentrated into 120 mM KCl, 20 mM Hepes, pH 7.5 with 200 μM PMSF on Centricon C30 cut-off filters (Amicon Ltd, Gloucestershire, U.K.). The final protein concentration of these extracts varied between 10 and 150 mg/ml. They were aliquoted, frozen in liquid nitrogen and stored at -80 °C. Boar sperm extracts that were used in these experiments were confirmed to be active in triggering Ca^{2+} oscillations when injected into mouse eggs (data not shown). The more concentrated boar sperm extracts needed dilution into 120 mM KCl, 20 mM Hepes, pH 7.5, so that when assayed in the sea urchin egg homogenate system a minimal concentration, that gave a maximal response, was achieved. Practically this meant the final protein concentration of the diluted sperm extracts varied between 10 and 30 mg/ml. This range is the same as that we have previously reported to be effective in homogenates [35,38]. Sperm extracts were also prepared from hamster and human. These extracts were made in the same manner as that described above for boar.

Tissue extracts were also prepared from boar brain, kidney, liver and testis. These tissues were washed and minced coarsely in PBS containing 200 μM PMSF and 2 $\mu\text{g}/\text{ml}$ leupeptin. They were homogenized on ice using an Ultra-Turrax T8 hand-held homogenizer. Homogenized tissues were then ultracentrifuged at 100 000 g for 1 h and the supernatant concentrated on C30 cut-off filters (protocol is the same as for boar sperm extracts). The protein concentrations of the tissues were equalized to 50 mg/ml.

Preparation of sea urchin egg homogenates

Homogenates (2.5%) of unfertilized *Lytechinus pictus* eggs (Marinus Inc., Long Beach, CA, U.S.A.) were prepared as described previously [38]. Briefly the intracellular medium consisted of 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 $\mu\text{g}/\text{ml}$ oligomycin; 1 mM sodium azide; 3 μM fluo-3. Ca^{2+} loading of intracellular stores was achieved by incubation at 17 °C for 3 h. Fluorimetry was performed at 17 °C using 500 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter. Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 and 535 nm, respectively. Additions were made in 2–5 μl volumes, except where otherwise stated.

Recombinant PLCs and the assay for PLC specific activity

PLC γ 1 and PLC β 1 were prepared from transiently transfected COS cells as described previously [41]. Recombinant PLC δ 1 was prepared as described previously [42]. PLC γ 2 was expressed using baculovirus/Sf9 cells. We found that preparations of PLC β 1 and PLC γ 1 both contained contaminating amounts of InsP_3 that by itself caused Ca^{2+} oscillations in mouse eggs and release in homogenate (data not shown). The recombinant PLCs were therefore purified by FPLC on a Mono-Q column (PLC γ 1 and PLC β 1) or Mono-Q and/or heparin-Sepharose column (PLC γ 2). PLC activity of sperm extracts and purified PLC were performed on mixed micelles with a Ca^{2+} concentration, buffered using Ca^{2+} /N-hydroxyethylethylenediaminetriacetic acid ('CaHEDTA'), at 10 μM , as described previously [43]. In this assay, 1 unit of PLC corresponds to hydrolysis of 1 μmol $\text{PtdIns}(4,5)\text{P}_2/\text{min}$.

Collection, microinjection and Ca^{2+} recording of mouse eggs

Mouse eggs, from adult MF1 mice, were collected in medium M2, 15 h after the intraperitoneal injections of 7.5 units of pregnant mares' serum gonadotrophin and 5 units of human chorionic gonadotrophin, which were given 48–52 h apart. Cumulus cells were removed by brief incubation with 0.3 mg/ml hyaluronidase and the zona pellucida removed by incubation in acid Tyrode's solution pH 2.5. Eggs were loaded with the Ca^{2+} -sensitive dye Fura-Red AM or with Fura-PE3 AM as described previously [35]. Ca^{2+} recordings and microinjection were performed as detailed in [35].

RESULTS

Dose-response of boar sperm extract in sea urchin egg homogenate

We have shown previously that boar sperm extracts contain a soluble PLC, which generates InsP_3 , and so causes Ca^{2+} release, when added to sea urchin egg homogenate [38]. This PLC activity appears wholly responsible for the ability of sperm extract to release Ca^{2+} in the homogenate since we observed no

Figure 1 The Ca^{2+} release response to sperm extract addition in sea urchin egg homogenate

(a) With increasing sperm protein addition to the egg homogenate there is a corresponding increase in Ca^{2+} release. These additions were prepared from a single 120 mg/ml sperm extract batch, which was serially diluted such that 2 µl additions were always made to homogenate. (b) The rate of RFU rise (RFU/s) observed for a series of sperm protein additions to homogenate. RFU is relative fluorescence units.

effect on the cADPR of the nicotinic acid-adenine dinucleotide phosphate (NAADP) receptors. Figure 1a shows that the boar sperm PLC activity is dose dependent. When 7.5–30 µg sperm extract protein was added to the homogenate there was a latency of several minutes before a Ca^{2+} rise. This confirmed that Ca^{2+} release was not associated with InsP_3 or Ca^{2+} within the sperm extract, since both these agents give immediate rises in Ca^{2+} [44,45]. The rate of Ca^{2+} rise followed a typical log dose response (Figure 1b).

For subsequent experiments, unless stated otherwise, sperm extracts were added to homogenates at the minimal dose required to give maximal Ca^{2+} release. Usually this was 2 µl of a 30 mg/ml solution. All of the experiments described here used sperm extracts that were washed and concentrated on 30 kDa cut-off filters. Thus the PLC must be greater than 30 kDa. However, we also obtained similar results using sperm extracts washed and concentrated on 500 kDa cut-off filters. This result suggests that the sperm PLC may form part of a high molecular mass aggregate of greater than 500 kDa.

Inhibitors of PLC signalling

Our previous data had suggested that the relevant PLC causing Ca^{2+} release in homogenate was sperm-derived and not egg-derived [38]. Thus when sperm extracts were added to egg homogenate it was a sperm PLC and not an egg PLC that caused InsP_3 production. We used pharmacological approaches

in order to confirm that the PLC was from sperm. The amino-steroid U73122 is a potent inhibitor of $\text{PtdIns}(4,5)\text{P}_2$ -PLC in a number of cell types [46–48]. At concentrations of 100 µM when added to the homogenate U73122 had little effect on the response to sperm extract (Figure 2a, $n = 3$). A higher dose of 400 µM U73122 caused by itself a small but permanent rise in Ca^{2+} ($n = 6$) and at this concentration it partially inhibited the response to sperm extract (Figure 2b). Higher concentrations were not examined because of the ability of 400 µM U73122 to raise the resting Ca^{2+} level. Thus preincubating homogenates with U73122 was ineffective at blocking the sperm extract response. We therefore examined the effect of preincubating the sperm extracts with U73122 since we had previously argued that the PLC was in the sperm not the egg. When sperm extracts were incubated with 400 µM U73122 ($n = 5$) it completely inhibited the Ca^{2+} -releasing ability of the extract in the homogenate (Figure 2c). In addition, 100 µM U73122 was effective at inhibiting sperm extract in 3 of 5 experiments. In control experiments the inactive analogue U7343 had no effect at either 1 mM ($n = 3$) or 400 µM ($n = 3$). These data indicate that U73122 was more effective at inhibiting the sperm extract response following preincubation with sperm extract rather than with homogenate.

Neomycin inhibits PLC activity by binding to PtdIns lipids [19]. It was reasoned, therefore, that neomycin would be effective at inhibiting sperm extract when added to homogenate since $\text{PtdIns}(4,5)\text{P}_2$ is present in the sea urchin egg homogenate. This was found to be the case and when 1 mM neomycin ($n = 6$) was added to the egg homogenate it abolished the Ca^{2+} -releasing activity of the sperm extract (Figure 2d). The effect of neomycin was selective since it did not block an InsP_3 induced Ca^{2+} rise ($n = 3$, Figure 2e) or the Ca^{2+} rise after addition of cADPR or NAADP ($n = 3$, data not shown). This suggests that neomycin is reasonably selective at inhibiting the sperm extract-induced Ca^{2+} release in egg homogenates.

Evaluation of the Ca^{2+} -releasing ability of mammalian sperm extracts and tissue extracts in sea urchin egg homogenate

All the above experiments were carried out using sperm extracts prepared from boar. We similarly examined the ability of sperm extracts prepared from hamster to release Ca^{2+} in homogenates. Hamster sperm extracts, like boar, have previously been shown to induce Ca^{2+} oscillations in various intact mammalian eggs [28,30,31]. We confirmed that hamster sperm extracts also released Ca^{2+} at similar protein concentrations to boar sperm ($n = 4$, Figure 3a). Release was through the InsP_3 receptor, since the response was ablated by prior desensitization of the InsP_3 receptor using repeated doses of InsP_3 (Figure 3b). Similar to boar and hamster, we also found that human sperm extracts triggered Ca^{2+} release in the sea urchin egg homogenate ($n = 2$ preparations, data not shown). These data suggest that the ability of a sperm PLC to release Ca^{2+} in this system was not specific to boar.

Since many tissues contain different isoforms of PLC [49] it is possible that extracts from many tissues would release Ca^{2+} in sea urchin egg homogenates. However, this was not the case. Tissue extracts prepared from boar brain ($n = 4$), kidney ($n = 3$) or liver ($n = 3$) did not show any Ca^{2+} release following addition to egg homogenate (Figure 3c), despite recording for extended periods. In contrast some weak Ca^{2+} -releasing activity was observed for whole testis extract ($n = 9$, Figure 3c). The protein concentrations of these tissue extracts were similar to concentrations of sperm extract that caused maximal release and therefore were directly comparable.

Figure 2 Inhibition of the sperm PLC with U73122 and neomycin

U73122 was more effective at inhibiting the response to boar sperm extract (SE) when preincubated with SE rather than homogenate. (a) Addition of 100 μ M U73122 to the homogenate does not inhibit the SE response but, (b) 400 μ M U73122 had some effect with the rate of rise and peak RFU decreasing while the latency increased. However note that 400 μ M U73122 raised the resting Ca^{2+} level. (c) Incubation of SE with 400 μ M U73122 for 30 min ablated the Ca^{2+} -releasing ability of SEs. (d) Preincubation of homogenates with 1 mM neomycin ('neo') blocked the response of homogenates to 2 μ l of 10 mg/ml SE. (e) Note that neomycin did not inhibit the Ca^{2+} -releasing ability of 400 nM InsP_3 when added to homogenate. Thus neomycin was a much better inhibitor of the SE response than U73122 following addition to the homogenate.

Figure 3 Sperm and testis extracts release Ca^{2+} in sea urchin egg homogenate but not tissue extracts from other sources

These experiments indicate that the ability of sperm extracts to release Ca^{2+} is not species specific to boar. (a) Hamster sperm extracts (HaSE) caused Ca^{2+} release when 2 μ l of 70 mg/ml sperm protein were added to the homogenate. (b) Desensitization of the InsP_3 response by repeated application of 1 mM InsP_3 abolished the ability of HaSE to release Ca^{2+} . (c) Tissue extracts were prepared from boar brain, kidney, liver and testis. See Experimental section for details of preparation. All tissue extracts were diluted such that 2 μ l additions were made to the homogenate of a 50 mg/ml tissue extract. Brain, kidney and liver extract failed to cause any Ca^{2+} release whereas testis extract caused a Ca^{2+} rise with a short delay. Asterisks indicate time of addition.

Figure 4 Recombinant semi-purified PLCs used for microinjection into mouse eggs and addition to sea urchin egg homogenate

(a) SDS/PAGE and Coomassie Brilliant Blue staining of purified preparations of PLC δ 1 and PLC γ 2. Recombinant PLC δ 1 (specific activity about 800 units/mg) was prepared as described previously [42]. PLC γ 2 (specific activity about 100 units/mg) was expressed using baculovirus/Sf9 cells and further purified on a heparin-Sepharose and/or a Mono-Q column. (b) Western blot analysis of partially purified preparations of PLC γ 1 and PLC β 1 using specific antibodies. Extracts of transiently transfected COS cells were prepared as described previously [41] and the activities further purified on a Mono-Q column; specific activity of PLC γ 1 was 5 units/mg and PLC β 1 10 units/mg.

Figure 6 Boar sperm extracts (SE) and recombinant PLC microinjection into mouse eggs

Only boar sperm extract caused any Ca^{2+} response when microinjected into mouse eggs. (a) SE ($n = 5$), (b) PLC β 1 ($n = 5$), (c) PLC γ 1 ($n = 5$), (d) PLC γ 2 ($n = 3$), (e) PLC δ 1 ($n = 5$). Microinjection volumes were between 1 and 5% the egg volume. The concentrations and activities of the SE and PLCs used are detailed in the legend of Figure 5. Ca^{2+} was recorded with either Fura Red (a, b, d, e) or Fura-PE3 (c). The results obtained were independent of the Ca^{2+} dye used. R.U. is ratio unit (see Experimental section for details of dye excitation/emission wavelengths).

Figure 5 Boar sperm extracts (SE) and recombinant PLC addition to sea urchin egg homogenate

Only boar sperm extract caused any Ca^{2+} release when added to the sea urchin egg homogenate, since PLC β 1, γ 1, γ 2 and δ 1 were without effect. (a) Addition of 0.004 unit of SE (2 μ l of an 11 mg/ml solution that had a PLC activity of 0.18 U/mg). (b) 0.009 U PLC β 1 (2 μ l of a 0.45 mg/ml solution with 10 U/mg PLC activity). (c) 0.009 U PLC γ 1 (5 μ l of a 0.35 mg/ml solution with a 5 U/mg PLC activity). (d) 0.205 U PLC γ 2 (5 μ l of a 0.41 mg/ml solution with a 100 U/mg PLC activity). (e) 4.8 U PLC δ 1 (2 μ l of a 3 mg/ml solution with an 800 U/mg PLC activity). Asterisks indicate time of addition.

Specific activity of PLC in sperm versus purified PLCs

The above data suggest that only a sperm-derived PLC is effective at generating InsP_3 in sea urchin egg homogenates. To test this further we examined a number of recombinant PLCs. We firstly compared their specific activities to that of sperm extract and then determined if they were able to release Ca^{2+} in sea urchin egg homogenate or following microinjection into intact mouse eggs.

Figure 4 shows Coomassie Brilliant Blue staining of recombinant purified PLC γ 2 and PLC δ 1 and Western blot analysis of semi-purified preparations of PLC β 1 and PLC γ 1. For all isoforms a band of the expected molecular mass was observed. Using a mixed micelle preparation containing radiolabelled $\text{PtdIns}(4,5)\text{P}_2$ and at a Ca^{2+} concentration of 10 μM all of the PLCs had a measurable PLC activity. Their specific activities were: PLC β 1, 10 unit/mg; PLC γ 1, 5 unit/mg; PLC γ 2, 100 unit/mg and PLC δ 1, 800 unit/mg. Using the mixed micelle assay we calculated the PLC specific activity of boar sperm extract to be 0.18 U/mg, a figure much lower than the recombinant PLCs. In contrast to sperm extract (Figure 5a) no rise in Ca^{2+} was observed when any of the recombinant PLCs were added to the sea urchin egg homogenate (Figures 5b–e); despite the fact that we were adding a higher enzymatic PLC activity than that found in sperm extracts (2- to 1000-fold higher). Similarly these PLCs were without effect on intracellular Ca^{2+} following microinjection into mouse eggs (Figure 6). In contrast, sperm extract caused Ca^{2+} oscillations as we have previously reported.

DISCUSSION

Previous data had shown that a soluble PLC from boar sperm is capable of releasing Ca^{2+} in sea urchin egg homogenate. Here we find a similar PLC activity in hamster and human sperm. Activity

was also found in extracts prepared from hamster testis but not from brain, liver and kidney. These data together suggest that the ability of cell extracts to release Ca^{2+} in egg homogenates is sperm-specific. Consistent with the hypothesis that a sperm PLC causes InsP_3 production once added to the egg homogenate, we found that the sperm PLC was blocked by preincubation with the PLC inhibitor U73122. Furthermore the PtdIns binding agent neomycin blocked the sperm PLC when preincubated with egg homogenate. The ability of the sperm PLC to release Ca^{2+} in the sea urchin egg homogenate was not shared by various recombinant PLCs (PLC β 1, γ 1, γ 2 and δ 1). These PLC all had greater specific activity than boar sperm extracts yet failed to release Ca^{2+} in sea urchin egg homogenate. Similarly PLC β 1, PLC γ 1, PLC γ 2 and PLC δ 1 did not cause Ca^{2+} oscillations following microinjection into mouse eggs. We suggest these data together imply that sperm PLC possesses distinct characteristics allowing it to hydrolyse endogenous egg PtdIns(4,5) P_2 .

A number of studies have indicated that increased PtdIns turnover and InsP_3 production occurs at fertilization in non-mammalian eggs [15–17,50]. Consistent with these PtdIns changes it has been shown that inhibiting Ca^{2+} release through the type I InsP_3 receptor, using a functionally inhibitory antibody, blocks the Ca^{2+} changes observed at fertilization in mouse [51]. However, the InsP_3 receptor may be triggered to release Ca^{2+} independent of a rise in intracellular InsP_3 [52]. Strictly therefore, in mammals direct evidence that InsP_3 production is involved in Ca^{2+} release at fertilization relies upon the inhibitory effects of the PLC inhibitor U73122 [18], whose action has been reported to have non-specific effects [21,53]. In our studies, when added to the homogenate, at high micromolar concentrations U73122 was ineffective at inhibiting the response to sperm extract but was effective when preincubated with the sperm extract. Since the PLC activity is in the sperm extract this would suggest a direct interaction of U73122 with the PLC. Although the mechanism of action of U73122 in inhibiting PLC is unresolved, it probably involves direct electrophilic attack on proteins through its very reactive maleimide group [17]. Our observations would be consistent with a direct inhibition of U73122 on PLC. Neomycin, in contrast to U73122, was effective at blocking the sperm extract-induced Ca^{2+} release when added to the homogenate, probably because it binds PtdIns(4,5) P_2 directly, the substrate of PLC. The inhibition by neomycin in homogenates is also consistent with its ability to block the sperm-induced Ca^{2+} wave at fertilization in intact sea urchin eggs [20]. On the basis of the present data, we suggest that it is possible that both U73122 and neomycin may inhibit Ca^{2+} release at fertilization [18,20] through an effect on a sperm-borne soluble PLC activity, rather than via a receptor-stimulated egg PLC.

Still to be addressed is whether the character of the sperm PLC that releases Ca^{2+} in sea urchin egg homogenate [38] and mouse eggs [35] is distinct from PLCs present in other tissues. Our current data have indicated that the Ca^{2+} -releasing activity is present in hamster, human as well as boar sperm extract, showing it is not species specific. This is consistent with previous findings in intact eggs, where the ability of hamster, human and boar sperm extracts to cause Ca^{2+} oscillations is not restricted to homologous eggs [26–31]. It seems likely that the PLC activity, which causes Ca^{2+} release in the homogenate, is not expressed outside the testis, since tissue extracts prepared from boar brain, liver and kidney, that would be expected to contain PLCs [49], lack any ability to trigger Ca^{2+} release. Again this is consistent with studies in intact eggs, where brain and liver tissue extracts have no ability to trigger Ca^{2+} oscillations [24,26]. Here we have found that various purified PLCs are not capable of causing Ca^{2+} release in sea urchin egg homogenates or intact mouse eggs.

Together with the observations using different tissues, which contain PLCs [49], these data clearly suggest that sperm possess either a novel type of PLC, or else a known PLC which is sperm-specifically regulated in a manner allowing it to generate InsP_3 in eggs. Thus, the ability of sperm extract to release Ca^{2+} in sea urchin egg homogenates and intact mouse eggs is not due merely to a non-specific high PLC activity.

It is not clear why the sperm PLC is able to cause InsP_3 production and Ca^{2+} release in the egg whilst other PLCs are not active (recombinant PLCs; brain, liver and kidney PLC). The recombinant PLCs we used have been shown to have PLC activity in cell lines, with PLC δ 1 being the most Ca^{2+} sensitive [41]. It is our working hypothesis that the sperm PLC possesses unique characteristics allowing it to target PtdIns(4,5) P_2 present in eggs. It may be relevant that the boar sperm PLC is active at around the 80 nM resting Ca^{2+} concentration present in our sea urchin egg homogenate (Rice, Jones & Swann, unpublished observation), whilst many PLCs require Ca^{2+} concentrations in excess of 1 μM to show significant activity [41,54].

Our previous data suggested that the sperm PLC is associated with the ability of sperm extracts to cause Ca^{2+} oscillations and egg activation in intact mammalian eggs [35]. Therefore, we proposed that this sperm PLC activity is the sperm factor responsible for fertilization. Here we have characterized this PLC activity further and demonstrated sperm-specific release in homogenate. The sperm PLC appears to possess novel characteristics not seen with PLCs from other sources or with recombinant PLCs with a greater specific activity.

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