Phospholipase C isoforms in mammalian spermatozoa: potential components of the sperm factor that causes Ca^{2+} release in eggs

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Injection of a soluble protein factor from mammalian spermatozoa triggers Ca^{2+} oscillations in mammalian eggs similar to those seen at fertilization. This sperm factor also generates inositol 1,4,5-trisphosphate and causes Ca^{2+} release in sea urchin egg homogenates and frog eggs. Recent studies have indicated that the sperm factor may be an inositol-specific phospholipase C (PLC) activity. This study investigated whether any of the commonly known PLC isoforms are components of the sperm factor. PLC β , PLC γ and PLC δ isoforms were shown to be present in boar sperm extracts. However, upon column fractionation of sperm extracts, none of the PLC isoforms detected

correlated with the ability to cause Ca^{2+} release in eggs. In addition to our previous work on recombinant PLCs, it was also shown that PLC δ 3, PLC δ 4 and its splice variant PLC δ 4 Alt1 fail to cause Ca^{2+} release. The recently discovered 255 kDa PLC ϵ isoform also appears unlikely to be a component of the sperm factor, as fractionation of sperm extracts on a gel filtration column demonstrated that the peak of Ca^{2+} -releasing activity was associated with fractions of 30–70 kDa. These findings indicate that the sperm factor that triggers Ca^{2+} release in eggs does not appear to have a known PLC isoform as one of its components.

Introduction

At fertilization, egg activation is triggered by a sperm-induced increase in intracellular Ca^{2+} (Nuccitelli, 1991; Whitaker and Swann, 1993; Stricker, 1999). In all vertebrate eggs and in eggs of many other species, the Ca^{2+} increase is a result of Ca^{2+} release from intracellular stores (Stricker, 1999). Phosphatidylinositol signalling is implicated in this process, as increased inositol 1,4,5-trisphosphate (InsP₃) production occurs at fertilization in sea urchin and frog eggs (Turner *et al.*, 1984; Nuccitelli, 1991), whereas blocking InsP₃ receptors in hamster and mouse eggs inhibits Ca^{2+} release at fertilization (Miyazaki *et al.*, 1993). However, the mechanism by which the spermatozoon induces Ca^{2+} release in the egg remains unresolved.

One theory of egg activation proposes that a surface interaction between a sperm ligand and an egg receptor leads to stimulation of an egg PLC β or PLC γ via either a G protein or a tyrosine kinase pathway, respectively (Jaffe, 1990; Schultz and Kopf, 1995; Evans and Kopf, 1998). The finding that injection of SH2 domains of PLC γ can block Ca^{2+} release and egg activation in starfish, sea urchin and ascidian eggs indicates an essential role for an egg-derived PLC γ (Carroll *et al.*, 1997, 1999; Shearer *et al.*, 1999; Runft

and Jaffe, 2000). However, injection of SH2 domains or G protein inhibitors does not block Ca^{2+} release in frog or mouse eggs (Williams *et al.*, 1998; Runft *et al.*, 1999). The identities of the receptors that may link sperm binding to egg activation in vertebrate eggs also remain unknown (Wassarman *et al.*, 2001).

Another explanation of how eggs are activated at fertilization proposes that Ca^{2+} release is triggered by a soluble sperm factor that enters the egg at gamete fusion (Whitaker and Swann, 1993; Fissore *et al.*, 1998; Swann and Parrington, 1999; Parrington, 2001). This hypothesis is supported by the finding that injection of soluble sperm extracts from boars, hamsters or humans can trigger Ca^{2+} oscillations in mouse, hamster, human and cow eggs similar to those seen at fertilization (Swann, 1990, 1994; Homa and Swann, 1994; Palermo *et al.*, 1997; Wu *et al.*, 1997; Fissore *et al.*, 1998). There is also evidence for a sperm factor in marine worms (Stricker, 1997), ascidians (Kyojuka *et al.*, 1998; McDougall *et al.*, 2000; Runft and Jaffe, 2000) and newts (Yamamoto *et al.*, 2001). The sperm factor activity is effective across different animal phyla because sperm extracts from frogs and chickens trigger Ca^{2+} oscillations when injected into mouse eggs (Dong *et al.*, 2000) and boar sperm extracts trigger Ca^{2+} oscillations in eggs of nemertean worms (Stricker *et al.*, 2000). The sperm factor appears to be a protein or proteins that may be sperm-specific, as the

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ability to trigger Ca^{2+} oscillations in eggs is not found in extracts from other tissues (Swann, 1990; Stricker, 1997; Wu *et al.*, 1997; Parrington *et al.*, 2000). The molecular identity of the sperm factor protein is currently unknown. It has been suggested that it is made up of a variety of different proteins such as a 33 kDa glucosamine deaminase, a truncated form of the c-kit receptor, a perinuclear protein or a nitric oxide synthetase (Parrington *et al.*, 1996; Sette *et al.*, 1997, 1998; Kuo *et al.*, 2000). However, none of these candidates now seems likely to be a component of the sperm factor (Wolsoker *et al.*, 1998; Wu *et al.*, 1998; Parrington *et al.*, 1999; Hyslop *et al.*, 2001).

Recent studies on the mammalian sperm factor protein have made use of sea urchin egg homogenate, a cell-free system that undergoes specific Ca^{2+} release in response to intracellular Ca^{2+} -releasing agents including InsP_3 (Lee, 1997). The same factor that is active in causing Ca^{2+} oscillations in eggs is able to cause Ca^{2+} release in sea urchin egg homogenates and this release is associated with the production of InsP_3 (Jones, 1998; Parrington *et al.*, 1999). The sperm factor also causes Ca^{2+} release in frog eggs via the generation of InsP_3 (Wu *et al.*, 2001). Data from homogenates also show that the ability of fractionated sperm extracts to cause Ca^{2+} release in eggs correlates with a PLC activity that can hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2) at low Ca^{2+} concentrations (Parrington *et al.*, 1999; Jones *et al.*, 2000; J. Parrington and K. Swann, unpublished). This finding indicates that the sperm factor contains a PLC activity. This sperm PLC activity is two orders of magnitude greater than PLC activities in other tissues and appears to be present in sufficient amounts in a single spermatozoon to account for the Ca^{2+} release observed at fertilization (Rice *et al.*, 2000). The ability of the PLC to cause InsP_3 production in egg homogenates also indicates that it has distinctive properties (Jones *et al.*, 1998, 2000; Parrington *et al.*, 1999; Rice *et al.*, 2000).

Which PLC isoform constitutes the active component of the mammalian sperm factor needs to be addressed. There are now four known major classes of PLC (Rhee, 2001): $\text{PLC}\beta$ (with four subtypes), $\text{PLC}\gamma$ (with two subtypes), $\text{PLC}\delta$ (with four subtypes) and $\text{PLC}\epsilon$. $\text{PLC}\beta$ and $\text{PLC}\gamma$ isoforms have a molecular mass of 140–150 kDa, $\text{PLC}\delta$ isoforms are 85–90 kDa and the $\text{PLC}\epsilon$ isoform is 255 kDa. The $\text{PLC}\beta 1$, $\text{PLC}\gamma 1$ and $\text{PLC}\gamma 2$ isoforms have been detected in mouse spermatozoa (Walensky and Snyder, 1995; Dupont *et al.*, 1996; Tomes *et al.*, 1996; Mehlmann *et al.*, 1998). $\text{PLC}\delta 1$ appears to be expressed in mouse spermatogonia (Lee *et al.*, 1999) and some splice variants of $\text{PLC}\delta 4$ appear to be testes-specific (Lee and Rhee, 1996; Nagano *et al.*, 1999). The aim of the present study was to investigate whether one of the known PLC isoforms is the active component of the mammalian sperm factor. Immunoblot analysis was used to identify which PLC isoforms are present in mammalian sperm extracts. The presence of the different known PLC isoforms in mammalian sperm extracts and subfractions that cause Ca^{2+} release in mouse eggs and sea urchin egg homogenates was investigated. The Ca^{2+} -releasing propert-

ies of recombinant $\text{PLC}\delta 3$, together with $\text{PLC}\delta 4$ and one of its splice variants, were also assessed. Finally, fractionation by gel filtration was used to determine the molecular mass of the sperm PLC activity.

Materials and Methods

Reagents

All chemicals were from Sigma (Poole) unless stated otherwise. Rabbit polyclonal antibodies raised against peptides specific to $\text{PLC}\beta$ (1–4), $\text{PLC}\gamma$ (1 and 2), $\text{PLC}\delta$ (1 and 2) were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to $\text{PLC}\delta 4$ was a generous gift from K. Fukami (University of Tokyo, Tokyo). Anti-rabbit peroxidase-conjugated polyclonal antibody was from DAKO (Cambridge). Immunoblots were developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont). Chromatography was performed on an AKTA FPLC system with a MonoS column and a Superdex 200 gel filtration column (Amersham Pharmacia Biotech).

Preparation of spermatozoa and sperm extracts

Boar, mouse, rat and hamster spermatozoa were washed three times in PBS. Pelleted spermatozoa (centrifuged for 1 min at 2000 g) were measured for amount of protein and were used fresh for SDS-PAGE analysis. Boar, mouse, rat and hamster sperm extracts were prepared by a freeze-thaw method as described by Jones *et al.* (1998). Sperm extracts were frozen in liquid nitrogen and stored at -80°C .

SDS-PAGE and immunoblot analysis

Whole tissue and tissue extracts were prepared for immunoblot analysis by addition to SDS-PAGE sample buffer and boiling for 5 min before loading on to an SDS-PAGE gel. Protein concentrations were measured using a bicinchononic acid protein assay (Sigma, Poole). SDS-PAGE and immunoblot analysis were performed as described by Parrington *et al.* (1996, 1999). For the $\text{PLC}\delta 1$ immunoblot, an enrichment step was carried out before SDS-PAGE. In brief, protein was incubated with 50 μl heparin Sepharose in a total volume of 500 μl PBS for 2 h at 4°C . The beads were pelleted by centrifugation at 2000 g for 1 min and washed three times with 1 ml cold PBS. After the final wash, the drained beads were added to 4 $\times$ SDS-PAGE loading buffer and boiled for 10 min. After pelleting the beads, 15 μl of the supernatant was loaded on to a 10% SDS-PAGE gel and subjected to immunoblotting as described above. In all cases the immunoblots were developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Chromatographic fractionation

A MonoS column was loaded with 6.5 mg boar sperm extract in 250 mmol KCl l^{-1} , 20 mmol 2-(N-morpholino)ethanesulphonic acid (MES) l^{-1} , pH 6, and

eluted with increasing salt up to 1 mol KCl l⁻¹ to fractionate boar sperm extract. The most active fraction from the MonoS column was loaded on to a Superdex 200 gel filtration column in 150 mmol NaCl l⁻¹, 50 mmol sodium phosphate l⁻¹, pH 7.0, to ascertain the molecular mass of the sperm factor. An identical gel filtration fractionation was carried out with native molecular mass standards to calibrate the column. For fractionation of hamster sperm extract, a MonoS column was loaded with 5 mg hamster sperm extract in 100 mmol KCl l⁻¹ in the same buffer and eluted with increasing salt up to 1 mol KCl l⁻¹. In each case, fractions were pooled, concentrated on Centricon C-30 concentrators (Millipore, Watford), washed and adjusted to the same protein concentration of 20 mg ml⁻¹ by dilution with 120 mmol KCl l⁻¹ and 20 mmol Hepes l⁻¹, pH 7.5.

Ca²⁺ release assays

Unfertilized sea urchin (*Lytechinus pictus*) egg homogenates (2.5%) (Marinus Inc., Long Beach, CA) were prepared as described by Jones *et al.* (1998) using fluo-3 (3 µmol l⁻¹) fluorescence as an indicator of Ca²⁺ release. For all assays of Ca²⁺ release, 2 µl of each fraction, previously adjusted to the same protein concentration, was added to 0.5 ml samples of homogenate (Jones *et al.*, 1998; Parrington *et al.*, 1999). Mouse eggs were collected from superovulated female MF1 mice and maintained in M2 medium. Microinjections and Ca²⁺ changes were carried out as described by Swann (1990).

Recombinant proteins

Recombinant versions of PLCδ3, PLCδ4 and PLCδ4 Alt1 were created. A construct containing the PLCδ3 full-length cDNA was a generous gift from J. Knopf (Genetics Institute, Cambridge). RT-PCR of rat spermatogenic cell mRNA generated PLCδ4 Alt1 and Alt2 splice variants, together with normal PLCδ4 sequence. These were identified by sequence analysis (MWG Biotech, Milton Keynes). The PLCδ3 sequence was subcloned into a pGEX-2T vector (Amersham Pharmacia Biotech) so that it was expressed as a glutathione S-transferase (GST) fusion protein. The PLCδ4 sequences were cloned into a pMAL-2X vector (New England Biolabs, Beverly) so that they were expressed as maltose binding protein (MBP) fusion proteins. Further sequence analysis was used to verify that the PLC fusion constructs were in the correct frame. These constructs were transfected into BL21 *E. coli* cells (Invitrogen, Groningen) and protein expression was induced by treatment with isopropyl β-D-thiogalactopyranoside (0.2 mmol l⁻¹) and protein expressed at 18–20°C for 16–18 h. Lysates were prepared by sonicating the bacterial pellet in lysis buffer. In the case of the GST-PLCδ3 construct, the lysis buffer was PBS supplemented with 1 mmol EDTA l⁻¹, 1 mmol dithiothreitol (DTT) l⁻¹ and complete protease inhibitors (Roche, Lewes); in the case of the MBP-PLCδ4 constructs, the lysis buffer was 25 mmol Hepes l⁻¹, 200 mmol NaCl l⁻¹, 1 mmol EDTA l⁻¹, 1 mmol DTT l⁻¹, 2 mmol 4-(2-

aminoethyl)benzenesulphonyl fluoride l⁻¹ and complete protease inhibitors (Roche). Resuspended cells were sonicated in three 30 s bursts. Lysates were clarified by centrifugation at 12 000 *g* for 10 min. The recombinant proteins were purified according to the appropriate manufacturer's protocol. In brief, GST-PLCδ3 was purified by binding to glutathione-sepharose beads (Amersham Pharmacia Biotech) and elution with 10 mmol reduced glutathione l⁻¹ in 50 mmol Tris l⁻¹, pH 8, whereas the MBP-PLCδ4 proteins were purified by binding to amylose resin (New England Biolabs) and elution with PBS containing 10 mmol maltose l⁻¹. After purification, recombinant proteins were buffer-exchanged into 20 mmol Hepes l⁻¹, 120 mmol KCl l⁻¹, pH 7.5, using a Sephadex G-25 column (Amersham Pharmacia Biotech) and concentrated using a Vivaspinn 8 ml concentrator (Sartorius, Epsom) to a protein concentration of 8–10 mg ml⁻¹. Concentrated protein was divided into aliquots and stored at –80°C until required.

Results

Detection and assessment of PLCβ, PLCγ and PLCδ isoforms in mammalian spermatozoa and sperm extracts

Immunoblot analysis was used to identify which PLCβ and PLCγ isoforms are present in boar sperm extracts. Proteins of the expected molecular mass (140–150 kDa) were detected in sperm extracts with antibodies to PLCγ1 and PLCβ2 (Fig. 1a), whereas with an antibody to PLCγ2, a protein of the expected size was detected in spermatozoa but not in sperm extracts. All the antibodies detected proteins of the expected molecular mass in boar brain (Fig. 1b). In sperm extracts, proteins of lower than expected molecular mass were also detected. Most notably, antibodies to PLCβ1 and PLCβ4 reacted with proteins of approximately 115 kDa and 70 kDa, respectively (Fig. 1a). Sperm extracts were fractionated on a MonoS cationic exchange column and the presence of PLC isoforms in the separated fractions was assessed using immunoblot analysis (Fig. 2a) to determine whether any of the putative PLC isoforms correlated with ability to trigger Ca²⁺ release in eggs. The bulk of the protein in the sperm extract appeared in the flow through fraction (Fig. 2a, i) but this fraction had no Ca²⁺-releasing activity in intact eggs or egg homogenates (Fig. 2a, ii and iii). Ca²⁺-releasing activity was found only in fractions D and E (Fig. 2a, i and ii). Immunoblot analysis with antibodies specific to PLCβ1, PLCβ2, PLCβ4 and PLCγ1 revealed that the proteins recognized by these antibodies were found only in the inactive flow through and not in the active fraction D (Fig. 2b). These data show that the PLCβ and PLCγ isoforms that are present in mammalian spermatozoa are unlikely to form part of the sperm factor that triggers Ca²⁺ release in eggs.

The PLCδ isoforms were also investigated as candidate sperm factors. Immunoblot analysis with an antibody against PLCδ1 showed that an 85 kDa protein corresponding to full-length PLCδ1, and a related breakdown

Fig. 1. Presence of phospholipase C (PLC)β and PLCγ isoforms in boar spermatozoa and brain extracts. (a) Proteins in whole boar spermatozoa (S) and sperm extract (SE) separated on an 8% SDS-PAGE gel, immunoblotted and probed with antibodies against PLCβ(1–4) and PLCγ(1–2). Antibodies used for each blot are shown at the top. Molecular mass markers are shown on the left and expected molecular masses of PLCβ and PLCγ are indicated by the arrows on the right. (b) Immunoblot of the same PLC isoforms in boar whole brain lysate (B) and soluble brain extract (BE) were separated and blotted as above. Expected molecular masses of PLCβ and PLCγ are indicated by the arrow on the right. A similar profile of immunoreactivity was observed on four separate occasions.

product of 68 kDa, could be detected in brain, kidney, liver and testes extracts, but could not be detected in sperm extracts (Fig. 3a, i). A similar comparison using an antibody against PLCδ2 indicated the presence of a protein of the expected molecular mass (85 kDa) in testes, which appeared to be present in high concentrations compared with amounts in other tissues (Fig. 3a, ii). This protein was also present in soluble sperm extracts (Fig. 3a, iii). However, upon fractionation of boar sperm extracts in a manner similar to that shown earlier (Fig. 2a), the 85 kDa protein recognized by the antibody to PLCδ2 was found in the inactive flow through fraction A, and not in fraction D which contained Ca^{2+} -releasing activity (Fig. 3a, iii). A protein of approximately 18 kDa in fraction D that was recognized by the anti-PLCδ2 antibody was found by N-terminal protein sequencing to be unrelated to PLCδ2 (data not shown).

An antibody against PLCδ4 failed to recognize a protein of the expected molecular mass in boar sperm extract (data not shown). However, in mice, rats and hamsters, PLCδ4 was found to be expressed in brain and spermatozoa, and was highly expressed in mouse and rat spermatozoa compared with expression in hamster spermatozoa (Fig. 3b, i). Further analysis showed that the PLCδ4 protein in mouse and rat spermatozoa is 3 kDa larger than that in the brain (Fig. 3b, ii), indicating that the PLCδ4 in spermatozoa could be the Alt1 splice variant (Lee and Rhee, 1996). Hamster sperm extracts were used to determine whether the

Fig. 2. Distribution of phospholipase C (PLC)β and PLCγ isoforms in fractionated boar sperm extract. (a) Fractionation of boar sperm extract on MonoS column. (i) Protein profile (measured at absorbance of 280 nm) of boar sperm extract fractionated on MonoS column, eluted with increasing KCl gradient (dashed line). A–F represent pooled and concentrated fractions. Before the assays were performed, the concentrated fractions were adjusted to the same protein concentration of 20 mg ml⁻¹. (ii) Two microlitres of each fraction (A–F) was assayed for the ability to cause Ca^{2+} release in sea urchin egg homogenates. Arrows indicate time of addition. RFU: relative fluorescence units of fluo3 present in homogenate. (iii) Fractions A and D were assayed for their ability to trigger Ca^{2+} oscillations when microinjected into mouse eggs. Arrows indicate time of injection. Fluorescence ratio units (FRU) are for Fura PE3-AM. A similar response pattern was seen in three other experiments. (b) Immunoblot of PLCβ and PLCγ isoforms in MonoS fractions. Fifty micrograms of sperm extract fractions A and D from MonoS chromatographic separation were separated on an 8% SDS-PAGE gel, immunoblotted and probed with antibodies to PLCβ1, PLCβ2, PLCβ4 and PLCγ1. The positions of fractions A and D are marked at the bottom, together with an indication of whether the fractions were inactive (–) or active (+) in triggering Ca^{2+} oscillations in mouse eggs and Ca^{2+} release in sea urchin egg homogenates. Molecular mass markers are shown on the left. Expected molecular mass of PLCβ and PLCγ is indicated by the arrow on the right. A similar immunoreactivity profile was observed on four separate occasions.

presence of PLCδ4 was associated with Ca^{2+} -releasing activity. Immunoblot analysis indicated that PLCδ4 was present only in the membrane pellet and not in the soluble fraction (Fig. 4a). Fractionation of hamster extract on a MonoS column showed that none of the lower molecular

Fig. 3. Presence of phospholipase C (PLC) δ isoforms in spermatozoa and testes compared with other tissues. (a) (i) Immunoblot of PLC δ 1 in boar tissues. An enrichment step was carried out before SDS-PAGE. Protein was incubated with 50 μ l heparin sepharose for 2 h and then washed. The beads were added to 4 $\times$ SDS-PAGE loading buffer and boiled for 10 min. After the beads were pelleted, 15 μ l of the supernatant was loaded on to a 10% SDS-PAGE gel, immunoblotted and probed with antibody to PLC δ 1. Boar brain (B), kidney (K), liver (L), sperm (S) and testes (T) soluble extracts are indicated. Molecular mass markers are shown on the left. Expected molecular mass of PLC δ 1 is indicated by the arrow on the right. A similar immunoreactivity profile was observed on two separate occasions. (ii) Immunoblot of PLC δ 2 in boar brain (B), kidney (K), liver (L) and testes (T) soluble extracts. In each case, 50 μ g of sample was separated on a 10% SDS-PAGE gel, immunoblotted and probed with antibodies to PLC δ 2. Molecular mass markers are shown on the left. Expected molecular mass of PLC δ 2 is indicated by the arrow on the right. A similar immunoreactivity profile was observed on four separate occasions. (iii) Immunoblot of boar sperm extract (BSE) and MonoS fractions (A and D) from Fig. 2a. In each case, 50 μ g of sample was separated on a 10% SDS-PAGE gel, immunoblotted and probed with antibodies to PLC δ 2. The position of fractions A and D is marked at the bottom, together with an indication of whether fractions were inactive (–) or active (+) in triggering Ca^{2+} oscillations in mouse eggs and Ca^{2+} release in sea urchin egg homogenates. Molecular mass markers are shown on the left. Expected molecular mass of PLC δ 2 is indicated by the arrow on the right. A similar immunoreactivity profile was observed on four separate occasions. (b) (i) Immunoblot of PLC δ 4 in whole tissue extracts of brain (B), heart (H), kidney (K), liver (L), spermatozoa (S) and testes (T) from mice, rats and hamsters. Fifty micrograms of sample was separated on a 10% SDS-PAGE gel, immunoblotted and probed with antibody to PLC δ 4. Expected molecular mass of PLC δ 4 is indicated by the arrows on the right. (ii) Immunoblot of PLC δ 4 in brain versus spermatozoa (whole tissue) in mice and rats. MB: mouse brain; MS: mouse spermatozoa; RB: rat brain; RS: rat spermatozoa. Arrows indicate positions of 90 kDa PLC δ 4 proteins and 93 kDa PLC δ 4 Alt 1 protein.

mass proteins recognized by the anti-PLC δ 4 antibody correlated with Ca^{2+} -releasing activity (Fig. 4b). These data, and those above, indicate that the PLC δ 2 and PLC δ 4 isoforms are not components of the sperm factor in mammalian spermatozoa.

Ca^{2+} -releasing properties of recombinant PLC δ 3 and PLC δ 4 isoforms

As a further test of the possibility that a known PLC may contribute to the sperm factor, the activity of recombinant PLC δ 3 and PLC δ 4 was tested in our cell-free system. It has already been shown that recombinant PLC β 1, PLC γ 1,

PLC δ 2 and PLC δ 1 fail to cause Ca^{2+} release in sea urchin egg homogenates (Jones *et al.*, 2000). In this study, recombinant PLC δ 3, PLC δ 4 and PLC δ 4 Alt1 proteins were prepared. An antibody to PLC δ 4 recognizes recombinant PLC δ 4 and PLC δ 4 Alt1; PLC δ 4 is 3 kDa larger than PLC δ 4 Alt1 (Fig. 5a). Although a diluted boar sperm extract caused Ca^{2+} release in homogenate, the recombinant PLC δ 3, PLC δ 4 and PLC δ 4 Alt1 failed to cause Ca^{2+} release (Fig. 5b,c). The lack of effect of recombinant PLCs was not due to lack of enzymatic activity because when the recombinant PLCs were pre-incubated with PIP_2 , and the homogenate used as an assay of IP_3 generation (Jones *et al.*, 1998), the recombinant PLC δ 3, PLC δ 4 and PLC δ 4 Alt1 had similar or

Fig. 4. Distribution of phospholipase C (PLC)δ4 in hamster sperm fractions. (a) Immunoblot of PLCδ4 in fractionated hamster sperm extract (SE), hamster sperm pellet (SP) and MonoS fractions A–F. Fractions fractionated on MonoS column, eluted with increasing KCl gradient (data not shown). A–F represent pooled and concentrated fractions. Before assays the concentrated fractions were adjusted to the same protein concentration of 20 mg ml⁻¹. Arrow indicates expected position of PLCδ4 protein. Molecular mass markers are shown on the right. (b) Two microlitres of hamster sperm extract and each MonoS fraction (A–F) was assayed for the ability to cause Ca²⁺ release in sea urchin egg homogenates. Arrows indicate time of addition. RFU: relative fluorescence units of fluo3 present in homogenate.

greater PLC activity than did the diluted boar sperm extract (Fig. 5c).

Size fractionation of boar sperm PLC activity

PLCε, for which antibodies were unavailable, has a molecular mass of 255 kDa, whereas other known PLC isoforms range in size from 85–90 to 140–150 kDa (Rhee, 2001). The molecular mass of the sperm factor was investigated by gel filtration chromatography. The active fraction D from a MonoS fractionation (Fig. 2a) was loaded

Fig. 5. Ca²⁺-releasing properties and phospholipase C (PLC) activity of boar sperm extract (BSE) and recombinant (r)PLCδ4 and rPLCδ4 Alt1. (a) Immunoblot of rGST, rPLCδ4 and rPLCδ4 Alt1. In each case, 1 μg of sample was separated on a 10% SDS-PAGE gel, immunoblotted and probed with an antibody to PLCδ4. (b) Addition of BSE (diluted 1:25), rPLCδ3, rPLCδ4 and rPLCδ4 Alt1 to homogenate. Two microlitres of each sample was assayed for the ability to cause Ca²⁺ release in sea urchin egg homogenates. Arrows indicate time of addition. RFU: relative fluorescence units of fluo3 present in homogenate. (c) Inositol 1,4,5-trisphosphate (InsP₃)-generating activity of BSE (diluted 1:25), rPLCδ3, rPLCδ4 and rPLCδ4 Alt1 measured in homogenate. Two microlitres of each of the same fractions was pre-incubated 1:1 with phosphatidyl-inositol 4,5-bisphosphate (PIP₂) for 1 min at room temperature (Ca²⁺ concentration fixed at 160 nmol l⁻¹ with Ca²⁺-EDTA buffer) and then 2 μl was added to the homogenate. Arrows indicate time of addition. RFU: relative fluorescence units of fluo3 present in homogenate.

on to a Superdex 200 gel filtration column and fractions were assayed for Ca²⁺-releasing activity in sea urchin egg homogenates. Upon comparison with native molecular mass standards, the peak of activity was found in a fraction corresponding to a molecular mass range of 29–70 kDa (Fig. 6b,c). A Coomassie-stained SDS-PAGE gel of the same fractions showed that most of the proteins eluted at the expected molecular mass (data not shown). This result is similar to that in a previous study, which found that the sperm factor active in causing Ca²⁺ oscillations in mouse eggs had a molecular mass in the range 29–70 kDa (Wu *et al.*, 1998).

Discussion

The results from the present study show that a number of PLCβ, PLCγ and PLCδ isoforms can be detected in mammalian spermatozoa. However, none of the common versions of the PLC subtypes detected in spermatozoa or

Fig. 6. Fractionation of semi-purified boar sperm extract by gel filtration. (a) Protein profile (measured at absorbance of 280 nm) of boar sperm extract previously fractionated on MonoS column (fraction D in Fig. 2a), fractionated on Superdex 200 gel filtration column. Fractions and elution point of native molecular mass standards (150, 66 and 29 kDa) are indicated. (b) Two microlitres of pooled, concentrated fractions were assayed for the ability to cause Ca^{2+} release in sea urchin egg homogenates. Fractions in each pool are indicated below each trace. Arrows indicate time of addition. RFU: relative fluorescence units of fluo3 present in homogenate. (c) Relative Ca^{2+} -releasing activity in homogenate of fractions from gel filtration. The estimated activity ratio was 1.00:0.33:0.04:0.00 for fractions 14–15:12–13:16–17:7–8.

sperm extracts appears to be a component of the soluble sperm factor that causes Ca^{2+} release in eggs. There are two lines of evidence that support this conclusion. Firstly, the known full-length isoforms of PLC do not correlate with the ability of extracts to cause Ca^{2+} release in intact eggs or in egg homogenates. Removal of all these isoforms in combination, through fractionation, also excludes the possibility that the sperm factor activity consists of these isoforms acting in tandem. These results from a wide set of PLCs are consistent with a recent study, which looked at a more limited subset of isoforms in spermatozoa (PLC γ 1, PLC γ 2 and PLC δ 4) and found no evidence that the isoforms correlated with the sperm factor activity in intact mouse eggs (Wu *et al.*, 2001). The PLC δ 4 isoform is also an

unlikely candidate for the mammalian sperm factor because spermatozoa from knockout mice that lack the PLC δ 4 isoforms (and its splice variants) possess Ca^{2+} -releasing activity (Fukami *et al.*, 2001). The other line of evidence against the known PLC β , PLC γ or PLC δ isoforms as a component of the sperm factor is that none of the common isoforms tested, in either this study or in previous reports (Jones *et al.*, 2000), is able to trigger Ca^{2+} release in eggs. The only isoform that was not tested in the present study is the newly described ϵ subtype of PLC (Song *et al.*, 2000; Kelley *et al.*, 2001; Rhee, 2001). This subtype of PLC has a molecular mass of 255 kDa, which is much greater than the apparent molecular mass (30–70 kDa) found for the sperm factor in the current study. This finding indicates that the PLC ϵ isoform is also unlikely to be a component of the mammalian sperm factor. With the caveat that some proteins elute differently from their predicted molecular mass, this gel filtration profile also indicates that the sperm factor has a molecular mass most similar to a PLC δ . It also makes it unlikely that the sperm factor is a complex of several PLCs.

There are several possible interpretations of the inability to correlate the sperm factor protein with any of the known isoforms of PLC. One possibility is that the sperm factor that causes Ca^{2+} release in mammalian eggs is an alternative type of enzyme (Kuo *et al.*, 2000; Runft and Jaffe, 2000; Wu *et al.*, 2001). However, this possibility is unlikely because the ability of sperm extracts to cause Ca^{2+} release in eggs is associated with the production of InsP_3 and with a highly active PLC that is present in sperm extracts (Jones *et al.*, 1998, 2000; Rice *et al.*, 2000). One interpretation of our current data is that mammalian sperm extracts contain an unidentified PLC activity. Whatever the nature of the sperm PLC activity, it is clearly distinctive in possessing an unusual capacity to cause Ca^{2+} oscillations in mouse eggs and Ca^{2+} release in sea urchin egg homogenates, which is not mimicked by recombinant versions of PLC β 1, PLC γ 1, PLC γ 2 and PLC δ 1 (Jones *et al.*, 2000). The recombinant PLC δ 3 and PLC δ 4 (together with its splice variant Alt1) also fail to mimic the properties of the sperm extract PLC activity.

If the sperm factor is a PLC, despite data from the current study, there are two more possible explanations. One is that the sperm PLC is a shortened or truncated form of a known PLC isoform that is not recognized by the panel of antibodies currently being used. Most of the antibodies react with peptides in the C-terminal region of the known PLCs and if this region were absent in a sperm-related form it would not be detected. Association of a truncated sperm PLC with other accessory proteins might also confer a difference in activity that could explain why the recombinant PLCs fail to cause Ca^{2+} release in eggs (Jones *et al.*, 2000). Future studies could address this possibility by using antibodies raised against internal regions of PLC isoforms. The other possibility is that sperm extracts contain a novel PLC isoform. This isoform could be sufficiently different from the known forms such that the current antibodies would fail to detect it. Such a novel PLC isoform would be

of a relatively small molecular mass (in the range close to 70 kDa) to be consistent with the current data.

The presence of a number of PLC isoforms in boar and rodent spermatozoa and testis raises the question of the function of the PLC isoforms in spermatozoa. PLC β 1 may be involved in the acrosome reaction, as a 115 kDa protein recognized by an antibody to PLC β 1 and G $_q$ and G $_{11}$ co-localizes to the acrosomal region of mouse spermatozoa (Walensky and Synder, 1995) and progesterone-stimulated diacyl glycerol (DAG) formation is not blocked by pertussis toxin (Murase and Roldan, 1996). Why PLC β 1 in mouse spermatozoa appears smaller than its expected molecular mass is not clear, but in the present study in boar spermatozoa a 115 kDa protein was detected with an antibody against PLC β 1. In brain extracts, the presence of a similar sized fragment of PLC β 1 was due to proteolysis by calpain (Park *et al.*, 1993). The acrosome reaction may also involve a PLC activated by a pertussis toxin-sensitive G protein (Go or G $_i$ type) as DAG formation triggered by zona pellucida is blocked by pertussis toxin (Murase and Roldan, 1996) and activation of G $_i$ has been demonstrated after stimulation of mouse spermatozoa with zona pellucida (Ward *et al.*, 1994). In this context, it is interesting that PLC β 2 has been found in boar spermatozoa, as activation of PLC β 2 by $\beta\gamma$ subunits may be an important route by which pertussis toxin-sensitive G proteins stimulate PLCs (Camps *et al.*, 1992; Katz *et al.*, 1992).

Previous studies of the role of phosphatidylinositol signalling proteins in sperm processes have tended to focus on the PLC β and PLC γ isoforms. The finding in the present study that PLC δ 1 is apparently present in high concentrations in testes extract but not in sperm extract is in line with a previous immunohistochemical study in which PLC δ 1 was found to be expressed in the spermatogonia (Lee *et al.*, 1999). The presence of PLC δ 2 at high concentrations in spermatozoa compared with those in other tissues is a novel finding and indicates a specific function. Data showing that rodent spermatozoa contain the PLC δ 4 splice variant PLC δ 4 Alt1 also indicate a specialized sperm-specific role for this isoform. Lee and Rhee (1996) reported that the Alt1 isoform is testes-specific, but it was not clear whether the protein was present in the spermatozoa, another type of testicular cell or in a type of cell at an earlier stage of spermatogenesis. A recent study detected only the Alt2 isoform in mouse spermatozoa, which appeared to be involved in the acrosome reaction (Fukami *et al.*, 2001). It is possible that spermatozoa contain several PLC δ 4 splice variants that fulfil different functions.

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