RESEARCH COMMUNICATION

The soluble sperm factor that causes Ca^{2+} release from sea-urchin (*Lytechinus pictus*) egg homogenates also triggers Ca^{2+} oscillations after injection into mouse eggs

John PARRINGTON*[1], Keith T. JONES*[1], F. Anthony LAI† and Karl SWANN*[2]

*Department of Anatomy, University College London, Gower Street, London WC1E 6BT, U.K., and †University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K.

Cytosolic extracts of boar sperm contain a soluble phospholipase C (PLC) activity that induces Ca^{2+} release in sea-urchin (*Lytechinus pictus*) egg homogenates and an uncharacterized protein factor that causes Ca^{2+} oscillations when injected into mammalian eggs. In the present study we fractionated boar sperm extracts on three different FPLC chromatographic columns and found that the fractions that caused maximal Ca^{2+} release in sea-urchin egg homogenates were also the ones that triggered Ca^{2+} oscillations in mouse eggs. Our data suggests that the sperm factor which triggers Ca^{2+} oscillations in eggs contains a PLC and not the 33 kDa glucosamine deaminase previously suggested to be one its components.

Key words: calcium, phospholipase C.

INTRODUCTION

At fertilization the sperm activates the egg by causing a single rise, or else a series of oscillations, in the cytoplasmic Ca^{2+} concentration [1,2]. The mechanism by which the sperm triggers Ca^{2+} release in eggs has not been established. The idea that sperm introduce Ca^{2+} directly into the egg seems unlikely in mammals [3]. Another hypothesis is that Ca^{2+} release is triggered by a surface-mediated interaction between sperm and egg [4]. This interaction may lead to the stimulation of a phospholipase C (PLC) within the egg, generating the Ca^{2+}-releasing messenger Ins(1,4,5)P_3. This is consistent with the finding of increased polyphosphatidylinositol lipid turnover and Ins(1,4,5)P_3 production at fertilization in sea-urchin and frog eggs [5–8]. Blocking Ins(1,4,5)P_3 receptors, or inhibiting PLC activity, also inhibits Ca^{2+} release at fertilization [2,8,9]. However, the molecular nature of the ligands that might couple to a PLC in the egg remain unclear.

A different hypothesis for egg activation suggests that Ca^{2+} release is triggered by a soluble cytosolic sperm factor that is introduced after the fusion of gametes [10–12]. This is supported by the finding that the injection of cytosolic sperm extracts can induce Ca^{2+} oscillations and other signs of egg activation in the eggs of hamsters, mice, humans, cows, nemertean worms and ascidians (sea-squirts) [10–17]. The injection of a single intact sperm into mouse eggs also induces Ca^{2+} oscillations similar to those seen at fertilization [18]. The sperm factor responsible for these effects is of high molecular mass and is both trypsin-sensitive and heat-labile, suggesting that it is proteinaceous in nature [10–12]. The protein factor appears to be highly conserved in mammals, as sperm extracts can induce Ca^{2+} oscillations in heterologous eggs [10,12–16].

The soluble sperm factor has not been identified. One candidate molecule is a 33 kDa glucosamine-6-phosphate deaminase from hamsters that was correlated with Ca^{2+}-oscillation-inducing activity over a series of chromatographic steps [19]. However, the mammalian 33 kDa glucosamine-6-phosphate deaminase does not cause Ca^{2+} oscillations when injected into eggs [20–22]. Another suggested candidate for a soluble sperm factor is tr-kit, a truncated product of the *c-kit* gene [23], but it is not known whether tr-kit causes Ca^{2+} oscillations in eggs.

We have recently shown that boar sperm extracts cause Ca^{2+} release from sea-urchin egg homogenates [24,25]. The sperm-extract-induced Ca^{2+} release in sea-urchin egg homogenates is due to the production of Ins(1,4,5)P_3 by a sperm-derived PtdIns(4,5)P_2-hydrolysing PLC activity [25]. This finding raises the possibility that the Ca^{2+}-oscillation-inducing activity of sperm extracts in intact egg is due to the same PLC activity which is active in sea-urchin egg homogenates. However, this possibility has not as yet been investigated. In the present study we fractionated boar sperm extracts using three chromatographic columns under stringent loading conditions. Fractions were assayed for Ca^{2+}-releasing activity in both intact mouse eggs and sea-urchin (*Lytechinus pictus*) egg homogenates and the presence of the 33 kDa glucosamine deaminase was detected by immunoblot. Our results suggest that the same sperm factor is active in the two systems and that Ca^{2+} oscillations in intact mammalian eggs may be stimulated by a sperm-derived PLC.

EXPERIMENTAL

Boar sperm cytosolic extracts were prepared as described previously, frozen in liquid N_2 and stored at −80 °C [25]. Protein concentrations were measured using a bicinchoninic acid ('BCA') protein assay kit (Sigma). Chromatography was performed on an FPLC system with Mono Q, Mono P and a HiTrap Heparin column (Pharmacia). The Mono Q column was loaded with

Abbreviation used: PLC, phospholipase C.

[1] These authors contributed equally towards this work.

[2] To whom correspondence should be addressed (e-mail k.swann@ucl.ac.uk).

6.5 mg of boar sperm extract at 0 M KCl, pH 7.5, and the protein eluted with increasing salt up to 1 M KCl. The Mono P column was loaded with 7.8 mg of sperm extract in 25 mM Bis-Tris, pH 7.2, and eluted with 1:10 Polybuffer 7–4 (Pharmacia), pH 4. The heparin column was loaded with 6.6 mg of sperm extract at 0.1 M KCl, pH 7, and eluted with increasing salt up to 1 M KCl. In each case, fractions were pooled and concentrated on Centricon C-30 concentrators and washed with buffer containing 100 mM KCl to adjust them to the same salt concentration. Fractions were adjusted by dilution with KCl buffer so that all samples had the same protein concentration for each set of assays for a run on a particular column (see the Figure legends). All solutions contained 20 mM Hepes.

Unfertilized sea-urchin (*L. pictus*) egg homogenates (2.5%) (Marinus Inc., Long Beach, CA, U.S.A.) were prepared as described previously using fluo3 (3 μM) fluorescence as an indicator of Ca^{2+} release [24,25]. For all assays of Ca^{2+} release, 2 μl of every fraction, previously adjusted to the same protein concentration, was added to 0.5 ml samples of homogenate [25]. Mouse eggs were collected from super-ovulated female MF1 mice and they were maintained in M2 medium as described previously [16,26]. Ca^{2+} changes in mouse eggs were monitored by the excitation fluorescence ratio of fura red (440 nm/490 nm) or fura-2 PE3 (350nm/380 nm). Eggs were loaded by 15 min incubation in 2 μM of the acetoxymethyl forms of fura red (Molecular Probes) or fura2-PE3 (Sigma). The measurements of fluorescence and microinjection of individual mouse eggs was performed as described previously [10,26]. For any one fraction to be assayed, at least two different eggs were injected for any one column and at least three eggs were injected when the flow-through fractions were being assayed for activity. Immunoblot analysis was used to detect the 33 kDa glucosamine-6-phosphate protein, using a 1:1000 dilution of a polyclonal antibody raised against the C-terminal sequence [19,22] and blotting and immunostaining conditions as described previously [19,22]. The blot was developed with the enhanced-chemiluminescence (ECL®) system (Amersham).

RESULTS AND DISCUSSION

To characterize the Ca^{2+}-releasing factor in sperm, a chromatographic separation of boar sperm extract was performed using three different chromatographic columns: Mono Q anionic exchange, Mono P cationic exchange and Heparin affinity. These were chosen for their differing modes of separation [27]. For each column the flow-through and eluted fractions were concentrated and adjusted to the same protein concentration. They were then assayed for the ability to induce Ca^{2+} oscillations when injected into mouse eggs and to cause Ca^{2+} release when added to sea-urchin egg homogenates. As reported previously [19], the activity of the sperm extracts was labile, so all assays were performed on the same day.

Figure 1(a) shows the fractionation of boar sperm extracts on a Mono Q column using increasing KCl concentration [19]. After injection into mouse eggs, clear Ca^{2+}-oscillation-inducing activity was found in two of the eluted fractions (Figure 1b) and weak Ca^{2+}-releasing activity in one other fraction (a single Ca^{2+} increase was seen in one of three eggs). When assayed by addition to sea-urchin egg homogenates the same two fractions that caused oscillations in mouse eggs were able to trigger Ca^{2+} release (Figure 1c). Furthermore, the rank order of potency in releasing Ca^{2+} in the sea-urchin egg homogenate indicated that the two most active fractions in homogenates caused Ca^{2+} oscillations in intact mouse eggs. The flow-through fraction, which contained protein that passed through the column, was inactive in causing

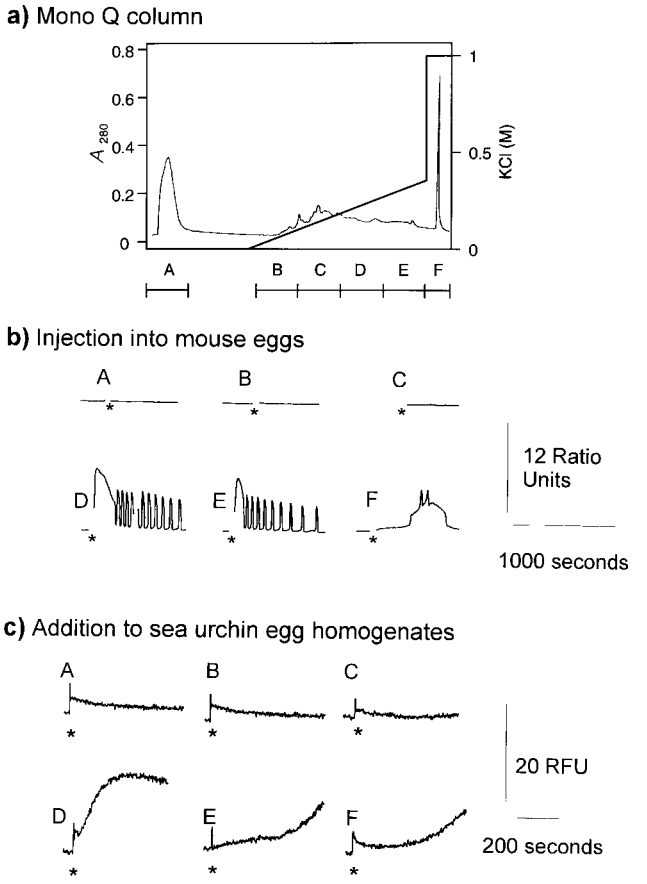

Figure 1 Fractionation of sperm extracts on a Mono Q column

In (**a**) is shown the A_{280} to indicate protein profile elution from the column due to an increasing KCl gradient (thick line). The capital letters (A–F) indicate the fractions that were pooled and assayed. Fraction A was the flow-through fraction that contained the most protein. Before assays all fractions were concentrated on ultrafiltration membranes and then adjusted by dilution to the same protein concentration (22 mg/ml). The fractions were assayed for their ability (**b**) to cause Ca^{2+} oscillations when microinjected into intact mouse eggs, and (**c**) to cause release in sea-urchin egg homogenates. In each trace of (**b**) and (**c**) the microinjections or addition were made at the time indicted by the asterisks, and the 'Ratio Units' in (**b**) are for PE3, and the RFU in (**c**) refer to relative fluorescence units of fluo3 present in the homogenates. By comparison with serial dilutions we estimated that the activity ratio in causing Ca^{2+} release in sea-urchin egg homogenates was 1:0.25:0.17 for fractions D, E and F respectively.

Ca^{2+} changes in either intact mouse eggs or sea-urchin egg homogenates. These data show that the factor which causes Ca^{2+} oscillations after injection into mouse eggs correlates with Ca^{2+} release in sea-urchin egg homogenates on this particular fractionation column.

Boar sperm extracts were next separated on a Mono P chromatofocusing column, which separates proteins according to their pI [27]. Figure 2(a) shows proteins eluted from the Mono P column using a gradient of pH decreasing from 7 to 4. Ca^{2+}-oscillation-inducing activity in mouse eggs was only found in two fractions that were eluted from the column (Figure 2b). These two fractions were clearly the most active in causing Ca^{2+}-release activity in sea-urchin egg homogenates (Figure 2c). The flow-through fraction was again inactive in both systems.

The ability of sperm extracts to cause Ca^{2+} release in sea-urchin egg homogenates is inhibited by heparin [25]. This may be partly because the relevant proteins in the sperm extract bind heparin, so the final separation method we used was fractionation on a heparin-affinity column [27]. In order to minimize non-specific binding, the sperm extract was again loaded at 100 mM

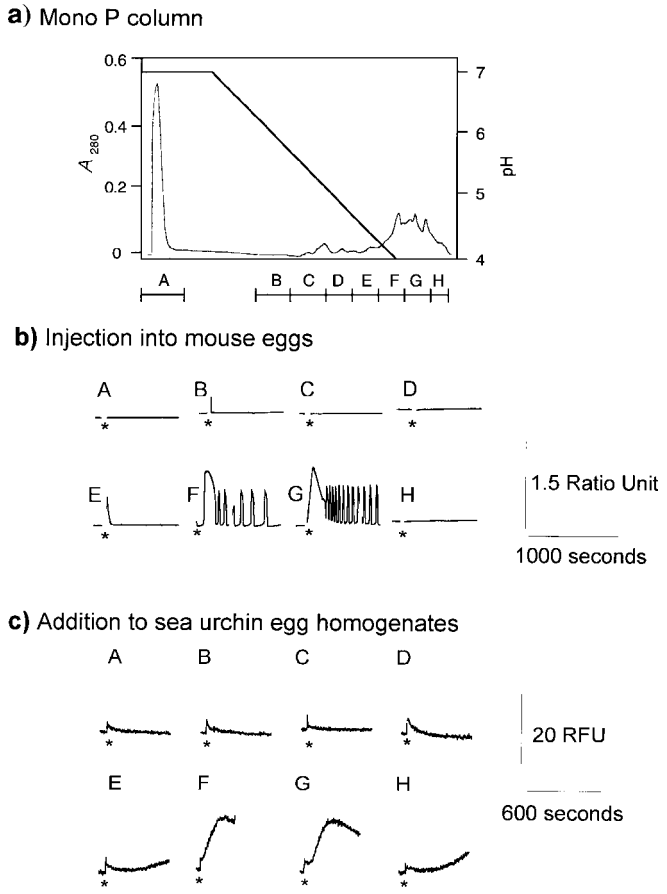

Figure 2 Fractionation of sperm extracts using a Mono P column

The conditions and protocol are as described in Figure 1. Fractions were eluted from the Mono P column by a decreasing pH gradient. The fractions that were separated were concentrated and adjusted to the same protein concentration (18 mg/ml) and labelled A–H before assays, with A being the flow-through fraction. In (**b**), fractions were microinjected into mouse eggs (Ca^{2+} monitored by fura red fluorescence), and in (**c**) fractions were added to sea-urchin egg homogenates. Each asterisk indicates the times of microinjection or addition. The estimated activity ratio in causing Ca^{2+} release in sea-urchin egg homogenates was 1:1:0.05:0.05 for fractions F, G, E and H respectively.

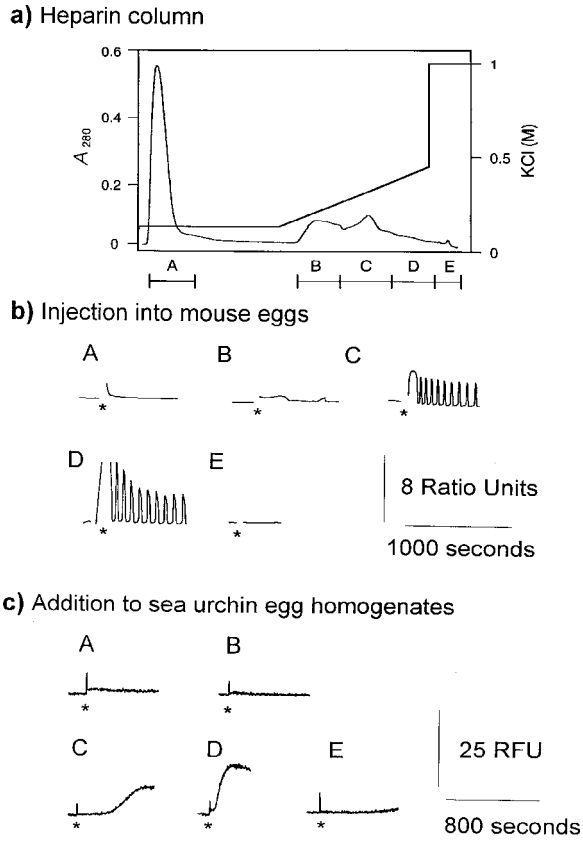

Figure 3 Fractionation of sperm extracts using a heparin-affinity column

The protocol and conditions were similar to those described in Figures 1 and 2. Fractions were eluted by increasing the KCl concentration, pooled, and adjusted to the same protein concentration (20 mg/ml); they were labelled A–D; the small peak labelled E contained 5 mg/ml protein. These were assayed in (**b**) by microinjection into mouse eggs (Ca^{2+} monitored using fura-2 PE3), and in (**c**) by addition to sea-urchin egg homogenates. The times of microinjection into eggs or addition to homogenates are indicated by asterisks. The estimated ratio of activities in causing Ca^{2+} release in sea-urchin egg homogenates was 1:0.20:0.064 for fractions D, C and E respectively.

KCl (Figure 3a). Ca^{2+}-oscillation-inducing activity in mouse eggs was found in two fractions eluted from the column (Figure 3b). Again these two fractions were also most active in causing Ca^{2+} release in sea-urchin egg homogenates, and the flow-through fractions was inactive in causing Ca^{2+} changes in either system (Figure 3c). The above experiments were reproduced with similar results in four separate trials for the Mono Q and Mono P columns and in three separate trials for the heparin-affinity column. We also found similar results using either Mono S or hydroxyapatite columns (results not shown). These results show a consistent correlation between the ability of sperm fractions to trigger Ca^{2+} oscillations in intact mouse eggs and the maximal activity of fractionated extracts to trigger Ca^{2+} release in sea-urchin egg homogenates.

We have previously used sequential fractionation methods, including Mono Q columns, to correlate the oscillation-inducing activity from hamster sperm with a 33 kDa protein termed 'oscillin' [19]. The recombinant 33 kDa protein does not cause Ca^{2+} oscillations by itself [20], but this did not exclude the possibility that it was associated with another protein that triggers Ca^{2+} release in eggs. We tested for the presence of the 33 kDa protein in the fractionated boar sperm extracts mentioned above. The immunoblot in Figure 4 shows that the active fractions from the Mono Q column and some of those active from the Mono P column contain the 33 kDa protein and Ca^{2+}-releasing activity (Figure 4a). However, with the heparin-affinity column the 33 kDa protein was detected only in the flow-through fraction (Figure 4c), which was inactive in causing Ca^{2+} release in sea-urchin egg homogenates or intact mouse eggs. These data support the notion that the 33 kDa protein does not correlate with Ca^{2+}-releasing activity and suggest that it is not a component of the sperm factor [20,21].

In a number of species a protein factor from sperm extracts induces Ca^{2+} oscillations similar to those seen at fertilization [10–17]. Our recent work demonstrated that boar sperm extracts contain a PtdIns(4,5)P_2-specific PLC activity that is responsible for causing Ca^{2+} release in sea-urchin egg homogenate [25]. The present results show that the maximal PLC that is active in homogenates co-fractionates, even under stringent conditions, with the Ca^{2+}-oscillation-inducing factor active in intact mouse eggs. As reported previously [25], we also found that all fractions active in sea-urchin egg homogenates had PLC activity when measured directly by incubation with PtdIns(4,5)P_2 (results not shown). This suggests that mammalian sperm contain a PtdIns-(4,5)P_2-specific PLC which is responsible for the sperm-factor activity that triggers Ca^{2+} oscillations and egg activation. Fur-

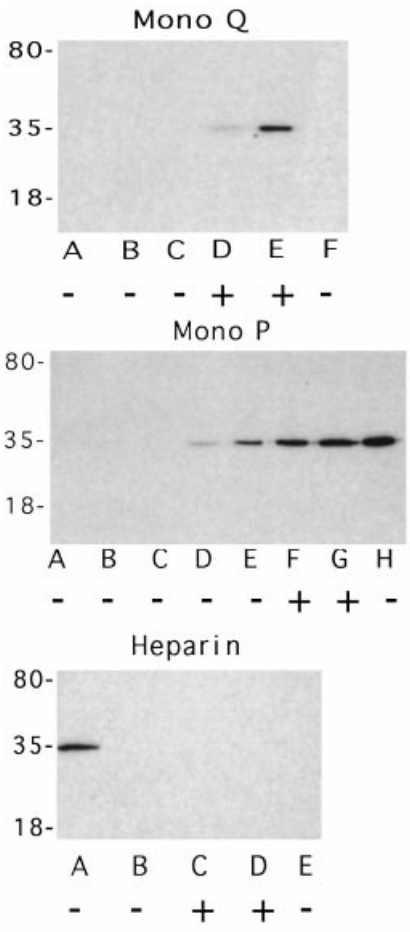

Figure 4 Immunoblot analysis of sperm extract fractions using an antibody against the 33 kDa glucosamine deaminase

Sperm-extract fractions obtained from chromatographic separation for the Mono Q column, for the Mono P column and for the heparin-affinity column were analysed by SDS/PAGE and then immunoblotted using a primary antibody (126) raised against the C-terminus of the 33 kDa protein. With each gel the position of molecular-mass markers are shown on the left-hand side. The various fractions A–F (from previous experiments in Figures 1–3) were run in the separate lanes indicated at the bottom. The fractions that showed both Ca^{2+}-oscillation-inducing activity in intact mouse eggs and peak Ca^{2+}-releasing activity in sea-urchin egg homogenates are indicated by a + sign in the bottom row; other fractions are labelled with a — sign.

thermore, since some fractions had weak activity in homogenates but failed to cause Ca^{2+} oscillations in eggs, it also appears that the sea-urchin-egg-homogenate system is more sensitive to the sperm-factor PLC than intact eggs. This greater sensitivity may be because the half-life for Ins(1,4,5)P_3 hydrolysis is 2–3 h in the sea-urchin egg homogenates [28] compared with a half-life of minutes to seconds in intact cells [7]. Previous experiments in mouse eggs showed that ryanodine inhibits sperm-factor-induced Ca^{2+} oscillations [29]. Since we now suggest that the sperm factor active in mouse eggs is a PLC that generates Ins(1,4,5)P_3, it seems likely that ryanodine is inhibitory because it depletes Ca^{2+} from a store that shares both Ins(1,4,5)P_3-sensitive and ryanodine-sensitive Ca^{2+}-release channels.

The molecular identity of the sperm PLC that appears to trigger Ca^{2+} release in either of our systems is not known. It may not be a common PLC because injection of tissue extracts from non-sperm cells does not cause Ca^{2+} oscillations in intact eggs [10,11,14,20] nor Ca^{2+} release in sea-urchin egg homogenates (J. Parrington, K. T. Jones, F. A. Lai and K. Swann, unpublished work). The relevant PLC must also be very sensitive to Ca^{2+}, since it is clearly active at the resting Ca^{2+} concentrations of $\approx$ 50 nM in sea-urchin egg homogenates [28]. Mammalian sperm extracts contain a PLC activity [30–32], but so far the γ1 and γ2 isoforms of PtdIns(4,5)P_2-specific PLC have been identified in whole sperm rather than soluble extracts [9,33]. In rat sperm homogenates a PtdIns(4,5)P_2-specific PLC activity was reported, but immunoblot analysis failed to detect PLC β1, γ1 or δ1 isoforms [34]. It will be important to establish which sperm PLC isoenzyme may trigger Ca^{2+} release in eggs and whether it requires other proteins from the sperm to stimulate its activity.

We thank Professor Bastien Gomperts and Dr. Jef Pinxteren for allowing us to use their FPLC apparatus, and Mr. Sam Alam for technical support. This work was supported by the Medical Research Council and the Wellcome Trust. J.P. is an MRC Research Fellow.

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Received 29 March 1999/9 April 1999; accepted 26 April 1999