Partial Characterization of the Calcium-Releasing Activity of Porcine Sperm Cytosolic Extracts

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Injection of sperm cytosolic extracts into mammalian eggs has been shown to elicit intracellular calcium ($[Ca^{2+}]_i$) oscillations that are similar in amplitude, duration, and frequency to those observed following fertilization. Thus, to characterize the Ca^{2+}-release component(s) in porcine sperm cytosolic extracts, a combination of fractionation techniques was used. The fraction with Ca^{2+} releasing activity was precipitated by 50% saturating solutions of ammonium sulfate and Western blot analysis showed that the pellets contained glucosamine-6-phosphate deaminase (gpd)/oscillin, a protein which has been suggested to be the sperm's active component. Single and double isoelectrofocusing (IEF) of porcine sperm extracts generated fractions with different Ca^{2+}-releasing activities. Fractions with maximal Ca^{2+}-releasing activity did not contain material that was immunoreactive with antibodies against gpd/oscillin; adjacent fractions containing gpd/oscillin had no Ca^{2+}-releasing activity. These findings were confirmed by IEF coupled with size exclusion chromatography on Superose 12 and with hydroxyapatite chromatography. These procedures predict an isoelectric point of our active component of 6.5–7.0 and a relative molecular weight ranging from 29 to 68 kDa. In summary, the data show that the Ca^{2+} release-inducing component(s) of porcine sperm extracts can be fractionated and that gpd/oscillin is not the pig sperm Ca^{2+} oscillogen.   © 1998 Academic Press

Key Words: sperm factor; $[Ca^{2+}]_i$ oscillations; mammalian eggs; oscillin; egg activation.

INTRODUCTION

Fertilization in mammalian species is characterized by the presence of long-lasting intracellular calcium ($[Ca^{2+}]_i$) rises (Miyazaki *et al.,* 1986; for review see Whitaker and Patel, 1990; Miyazaki *et al.,* 1993). These $[Ca^{2+}]_i$ oscillations are necessary to trigger egg activation and initiate embryonic development (Whitaker and Patel, 1990; Kline and Kline, 1992).

The mechanisms by which the sperm initiates Ca^{2+} release and the intermediate signaling molecules necessary for the persistence of $[Ca^{2+}]_i$ oscillations are not clearly elucidated (Whitaker and Swann, 1993). Two hypotheses have been formulated. The receptor hypothesis predicts the presence of a receptor in the egg plasma membrane with which the sperm must interact. Upon binding, this receptor is likely to have the capacity to initiate a signaling cascade that leads to the generation of 1,4,5-inositol trisphosphate (InsP3) and Ca^{2+} release (see Schultz and Kopf, 1995, for recent review). In support of this hypothesis, molecules involved in the production of InsP3, either through G-protein or tyrosine kinase-coupled receptors, have been demonstrated to be present in mammalian eggs (Miyazaki, 1988; Williams *et al.,* 1992, 1998; Dupont *et al.,* 1995). Although a specific sperm receptor responsible for $[Ca^{2+}]_i$ oscillations has not been found in the plasma membrane of eggs, it is possible that integrins, which are present in the surface of mammalian eggs, may serve this purpose (Fusi *et al.,* 1993; Almeida *et al.,* 1995; Bronson *et al.,* 1995; Evans *et al.,* 1995). Moreover, these molecules have potential signaling cytoplasmic domains and functions (Snell and White, 1996; Iwao and Fujimura, 1996).

The fusion hypothesis predicts that upon sperm–egg fusion a sperm cytosolic component is released into the egg's cytoplasm and initiates Ca^{2+} release by interacting

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with an egg molecule. Several studies support this last hypothesis including recent results using intracytoplasmic sperm injection (ICSI) (for review see Swann and Lai, 1997). ICSI studies have demonstrated that initiation of $[Ca^{2+}]_i$ oscillations and activation of mammalian eggs are possible without sperm/egg plasma membrane interaction (Palermo *et al.,* 1992; Tesarik *et al.,* 1994; Nakano *et al.,* 1997) and that these properties are sperm-specific since the ability to induce egg activation is present only in certain stages of spermatogenesis (Kimura and Yanagamachi, 1995) and injection of leukocytes into oocytes did not sensitize Ca^{2+} release as sperm cells did (Souza *et al.,* 1996). Additional support for the fusion hypothesis was provided by injection of sperm cytosolic extracts into eggs that has been shown to evoke $[Ca^{2+}]_i$ oscillations similar to those observed during fertilization (Dale, 1985; Swann 1990; Homa and Swann, 1994; Palermo *et al.,* 1997; Stricker, 1997; Wu *et al.,* 1997) and to initiate parthenogenetic development to the blastocyst stage (Stice and Robl, 1990; Dozortsev *et al.,* 1995; Wu *et al.,* 1998; Fissore *et al.,* 1998).

The Ca^{2+} release component(s) of these sperm extracts is not known (Galione *et al.,* 1997). Recently, a cytosolic protein isolated from hamster sperm has been suggested to be the active molecule because its presence correlated, after several fractionation procedures, with Ca^{2+}-releasing activity (Parrington *et al.,* 1996). This protein was named oscillin and was later identified as glucosamine-6-phosphate deaminase/isomerase (gpd) (Wolosker *et al.,* 1998). Although gpd/oscillin localizes to the equatorial segment of mammalian sperm (Parrington *et al.,* 1996), its role in mammalian fertilization has been recently questioned (Wolosker *et al.,* 1998). Truncated c-Kit, a shorter form of the c-kit receptor which is expressed in late stages of spermatogenesis, has also been postulated to be part of the sperm's cytosolic activating content (Sette *et al.,* 1997), although the Ca^{2+}-inducing activity of this molecule was not tested.

The present study was undertaken to characterize the active fractions in porcine cytosolic sperm extracts and to determine if the Ca^{2+}-releasing activity of these fractions contained gpd/oscillin. Our results, obtained by a combination of isoelectrofocusing (IEF) and chromatographic procedures, suggest that the active component(s) of porcine sperm cytosolic extracts may be a molecule(s) other than gpd/oscillin.

MATERIALS AND METHODS

Egg Recovery

Eggs were obtained from the oviducts of CD-1 female mice (6–12 weeks old) superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO) followed 48 h later by injection of 5 IU of human chorionic gonadotropin (hCG; Sigma) to induce ovulation. Eggs were recovered 14 h post-hCG into a Hepes-buffered solution (TL–Hepes, Parrish *et al.,* 1988) supplemented with 10% heat-treated calf serum (FCS; Gibco, Grand Island, NY). Cumulus cells were removed with bovine testes hyaluronidase (Sigma). For this study, eggs with the first polar body and no signs of degeneration were chosen. These eggs were placed in 50-μl drops of TCM-199 culture medium (Gibco) supplemented with 10% FCS (Gibco) under paraffin oil at 36.5°C in a humidified atmosphere containing 7% CO_2 until the time of injection.

Sperm Factor Preparation

Cytosolic sperm extracts were prepared from boar semen as previously described (Swann *et al.,* 1990; Wu *et al.,* 1997, 1998); boars were chosen over males of other species because they provide large amounts of sperm. Briefly, semen samples were washed twice with TL–Hepes medium and the sperm pellet was resuspended in a solution containing 75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM glycerophosphate, 1 mM DTT, 200 μM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, pH 7.0. The sperm suspension was sonicated for 25–35 min at 4°C (XL2020, Heat Systems Inc., Farmingdale, NY). The lysate was then spun twice at 10,000g and, in both cases, the supernatants were collected. The resulting supernatant was centrifuged at 100,000g for 1 h at 4°C and the clear supernatant was used as the cytosolic fraction. Ultrafiltration membranes (Centriprep 10 and 30 and Centricon 30, Amicon, Beverly, MA) were used to wash the supernatant (75 mM KCl and 20 mM Hepes, pH 7.0) and concentrate these extracts to final concentrations of 10–60 mg/ml protein.

Ammonium Sulfate Precipitation

Crude sperm extracts were mixed with saturated ammonium sulfate to 50% saturation, the precipitates were collected by centrifugation (10,000$g,$ 15 min, 4°C), and the pellets were stored at −20°C until use. Pellets were resuspended in injection buffer (75 mM KCl and 20 mM Hepes, pH 7.0), washed in the same buffer, and concentrated with ultrafiltration membranes before assaying for Ca^{2+}-releasing activity.

Isoelectric Focusing

Single IEF of active fractions obtained from ammonium sulfate precipitation was performed for 4.5 h at 4°C and 12 W with 2% ampholytes (pH 3–10) using a Rotofor Preparative IEF cell (Bio-Rad, Hercules, CA). Sperm extracts (60–80 mg total protein) were diluted in ddH$_2$O, subjected to IEF, pooled, washed with injection buffer, and concentrated by ultrafiltration before assaying for Ca^{2+}-releasing activity. For double IEF, active fractions from single IEF were pooled, supplemented with 2% ampholytes of narrower pH range (5–8) and IEF carried out for 3.5 h at 4°C and 12 W. The obtained fractions were pooled, washed, concentrated, and tested for Ca^{2+}-releasing activity.

Superose 12 FPLC Chromatography

The active fractions obtained from ammonium sulfate precipitation or IEF were loaded at 4°C onto a Superose 12 HR 10/30 column connected to a fast protein liquid chromatography (FPLC) system. Proteins were eluted with buffer (75 mM KCl and 20 mM Hepes, pH 7.0) containing 200 μM PMSF at a flow rate of 0.1 ml/min and detected at OD$_{280}$ by an UV-M monitor. Fractions (0.25 ml) were collected and concentrated before testing for Ca^{2+}-releasing activity. The Superose 12 HR 10/30 column was cali-

brated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), and carbonic anhydrase (29 kDa) (Sigma).

Hydroxyapatite FPLC Chromatography

Ammonium sulfate pellets were diluted into 10 mM potassium phosphate buffer (pH 6.8) + 200 μM PMSF and loaded at 0.4 ml/min onto a 5-ml hydroxyapatite column using FPLC system at 4°C. After a 10-ml wash with 10 mM phosphate buffer, proteins were eluted at the same flow rate by increasing the molarity of the potassium phosphate buffer (pH 7.2 + 200 μM PMSF) in a stepwise manner. The potassium phosphate concentration in each step was as follows: 88, 127, 185, 244, 302, and 400 mM. Fractions of 1.0 ml were collected, pooled, washed with injection buffer, and concentrated before testing for Ca^{2+}-releasing activity. In one experiment, the loading and elution buffer contained 200 μM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin. The presence of additional protease inhibitors did not modify the protein elution profile or the distribution or amount activity of the collected sperm fractions (not shown).

Protein Expression and Production of Polyclonal Rabbit IgG Specific for Recombinant Glucosoamine-6-phosphate Deaminase/Oscillin

Hamster sperm glucosamine-6-phosphate deaminase/isomerase (gpd)/oscillin cDNA (a generous gift from Dr. K. Swann and Dr. A. Lai, University College and MRC National Institute for Medical Research, London, UK) was subcloned into the glutathione S-transferase (GST)–gene-based expression vector pGEX 3 system (Pharmacia, Pitscataway, NJ). The correct orientation and position of the gpd/oscillin insert was confirmed by sequencing of nucleotides in the site of initiation of transcription (DNA Services, Cornell University, Ithaca, NY). The construct was then transformed into *Escherichia coli* BL21 strain, and GST–gpd/oscillin fusion protein expression was stimulated by addition of IPTG. The expressed GST fusion protein was purified by affinity chromatography using glutathione Sepharose 4B beads according to the manufacturer's instructions (Pharmacia). The gpd/oscillin fusion protein (Fig. 2) was first separated from its GST fusion partner by cleavage with the protease factor Xa (Pharmacia), and then the factor Xa was removed from the preparation by benzamidine Sepharose 6B beads (Pharmacia). Protein purity before injection into rabbits was checked by SDS–PAGE and Coomassie blue staining (not shown).

Purified recombinant hamster gpd/oscillin was injected into rabbits to produce polyclonal antibodies. The immunization procedure involved an initial injection (40 μg of recombinant gpd/oscillin) followed by two boost injections of 20 μg of recombinant protein 3–4 weeks apart. These procedures were carried out by the university's animal care antibody production group.

A second polyclonal antibody was generously shared by Dr. G. Palermo (Cornell Medical School, New York, NY). This antibody was raised against a conserved region of human gpd by injecting a peptide containing the following sequence: KYVKTFNMDEYVGL-PRDHPESYHSF. The antibody was subsequently affinity purified, eluted via a pH gradient, and stored in a borate buffer. Production and purification of this antibody was carried out by Research Genetics, Inc. (Huntsville, AL).

Western Blots

Proteins from sperm fractions were run in 12% SDS–PAGE and transferred to nitrocellulose membranes (Micron Separation, Westboro, MA) essentially as described for bovine eggs (He *et al.*, 1997). After blocking the membranes with 6% milk in 0.1% Tween 20, blots were incubated with 1:1000–4000 dilutions of the anti-gpd/oscillin polyclonal antibodies at 4°C for 1 h and then washed and incubated for 1 h at 4°C with a secondary horseradish peroxidase-coupled antibody. After several washes, membranes were developed using the ECL detection system according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

The presence of the truncated c-kit protein in our fractions was investigated by Western blotting using a polyclonal antibody against the c-terminal end of c-kit (Santa Cruz Biotechnology, Santa Cruz, CA).

Microinjection Techniques and [Ca²⁺]ᵢ Oscillation Monitoring

Microinjection procedures were as previously described (Wu *et al.*, 1997, 1998). In brief, eggs were microinjected using Narishige manipulators (Medical Systems Corp., Great Neck, NY) mounted on a Nikon Diaphot microscope (Nikon Inc., Garden City, NY). Glass micropipets were filled by suction of a microdrop containing 0.5 mM fura 2 dextran (fura 2D, dextran 10 kDa, Molecular Probes, Eugene, OR) or sperm extract (1–20 mg/ml protein concentration). Solutions were expelled into the cytoplasm of eggs by pneumatic pressure (PLI-100, picoinjector, Medical System Corp., NY). The injection volume was approximately 5 to 10 pl and resulted in final intracellular concentration of the injected compounds of approximately 1% of the concentration in the injection pipette.

Fura 2D fluorescence was monitored as previously described (Wu *et al.*, 1997, 1998). Briefly, excitation wavelengths were at 340 and 380 nm and the emitted light was quantified, after passing through a 500-nm barrier filter, by a photomultiplier tube. The intensity of excitation light was attenuated by neutral density filters, and the fluorescent signal was averaged for the whole egg. $[Ca^{2+}]_i$ concentrations, R_{min}, and R_{max} were calculated according to Grynkiewickz *et al.* (1985) and Poenie (1990) and as previously reported (Fissore and Robl, 1993; Wu *et al.*, 1997, 1998); R_{min} and R_{max} values were of 0.15 and 1.5, respectively.

$[Ca^{2+}]_i$ monitoring of mouse eggs started 30–45 min after injection of fura 2D, which was approximately 15 h post-hCG. Eggs were monitored individually in 50 μl medium placed on a glass coverslip on the bottom of a culture dish covered with paraffin oil. Fluorescence ratios were obtained every 4 s for 15 to 30 min depending on the experiment. Prior to the injection of sperm factor, fluorescent recordings were taken to establish baseline values. Readings were taken for 1 s at each wavelength. $[Ca^{2+}]_i$ monitoring was completed before eggs had reached 22 h post-hCG. All sperm extracts fractions were tested at 1 mg/ml protein concentration unless indicated. The activity of fractions was arbitrarily classified as follows: a − was used to indicate absence of activity; a + was used for fractions that initiated a first spike within 10–20 s after the injection but $[Ca^{2+}]_i$ raises did not last for 10 min; ++ was used for fractions that initiated oscillations that lasted for at least 10 min and showed four or less rises per 10 min; +++ was used for fractions whose Ca^{2+} responses lasted more than 20 min and four or more rises occurred per 10-min intervals. In this last group, the decline in activity was minimal for the first 20 min of monitoring. Assessment of the frequency of $[Ca^{2+}]_i$ rises for activity determination was conducted 5 min postinjection and for 10 min. In those
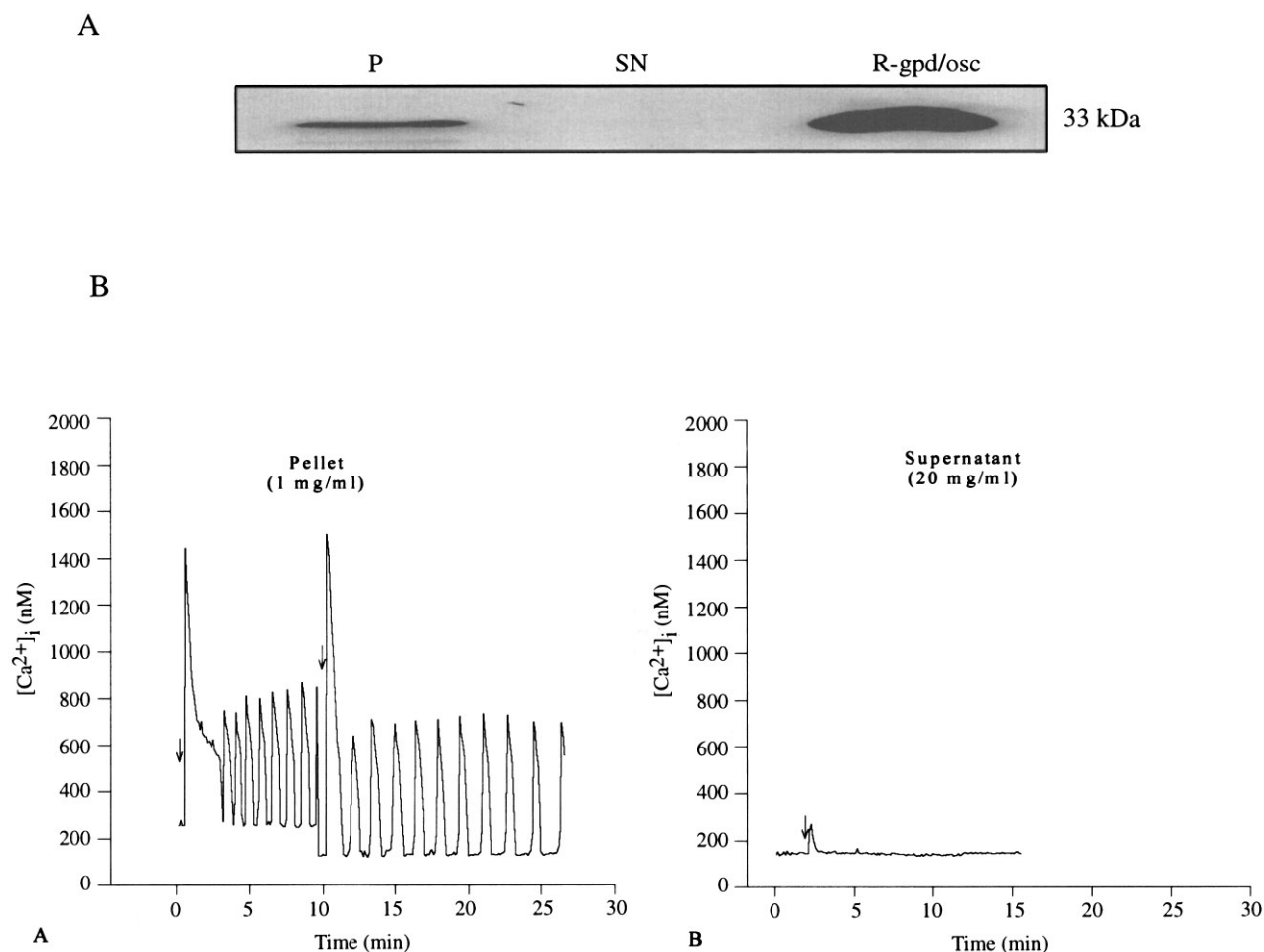
A

B

FIG. 1. Ammonium sulfate fractionation of porcine sperm extracts. Precipitates (P) and supernatants (SN) obtained by exposure to 50% saturating solutions were tested for the presence of immunoreactive gpd/oscillin (A) or for Ca^{2+} activity (B). P contained immunoreactive gpd/oscillin (A) and induced $[Ca^{2+}]_i$ oscillations when injected into mouse eggs (B, left). SN fractions had no immunoreactive gpd/oscillin and did not contain Ca^{2+}-release-inducing activity (B, right). Injections are denoted by an arrow and the pipet was removed immediately after the injection; each arrow indicates injection into a different/new egg. Recombinant gpd/oscillin (R-gpd/osc) was used as positive control in Western blotting analysis. Sperm fractions were loaded at 30 μg total protein per lane for all Western blot analysis and R-gpd/osc was loaded at 20 ng per lane. Precipitates were produced multiple times and their Ca^{2+} activity was tested at least three different times and at least two eggs were injected each time.

cases in which the first rise lasted for more than 5 min, activity determination was started as soon as the baseline was reestablished and then monitored for 10 min. At least two eggs per fraction were tested for Ca^{2+} activity per replicate; the number of replicates for each fractionation procedure are indicated on the figure legends. Within fractionation procedure, the profile of Ca^{2+} activity between replicates was highly consistent.

RESULTS

Ammonium Sulfate Fractionation of Porcine Cytosolic Sperm Extracts

Porcine sperm extracts were exposed to ammonium sulfate solutions to fractionate its active components and to accumulate them for further purification procedures. A 50% saturating solution consistently precipitated the Ca^{2+} releasing activity of porcine sperm extracts (Fig. 1B$_A$), while supernatants fractions exhibited no Ca^{2+} activity even when tested at high protein concentrations (> 20 mg/ml; Fig. 1B, right). Ca^{2+} release by these precipitates was unaffected by long storage (data not shown).

We then investigated if the active precipitated fractions contained gpd/oscillin. Western blotting analysis, using an antibody raised against the whole hamster gpd/oscillin sequence, showed a strong immunoreactive band of 33–35 kDa in the pellet fractions, which is the presumed size of the gpd/oscillin subunits; no band was observed in the supernatant fractions (Fig. 1A). The recombinant protein
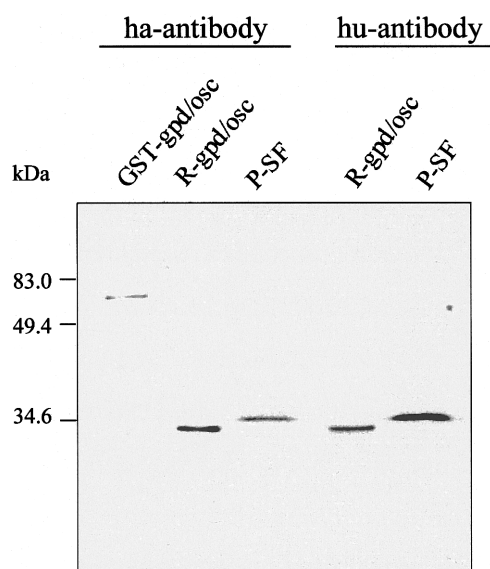
FIG. 2. Western blot analysis of gpd/oscillin from porcine sperm extracts. Two different antibodies, one raised against the whole hamster sperm molecule (ha-antibody) and the other raised against a conserved peptide of the human gpd/oscillin molecule (hu-antibody) were used to test the presence of gpd/oscillin (gpd/osc) in porcine sperm precipitates (P-SF). These antibodies also detected recombinant gpd/oscillin (R-gpd/osc). The intact fusion protein, GST–gpd/osc was identified by ha-antibody. Similar results were observed in three separate blots.

used to raise the antibody was run in parallel as positive control (Figs. 1A and 2) and our antibody also recognized the GST–gpd/oscillin fusion protein (Fig. 2). To confirm that our antibody was identifying gpd/oscillin, an additional polyclonal antibody was used in Western blotting procedures. This antibody was raised against a conserved internal sequence of human gpd/oscillin. This antibody recognized a band of similar molecular weight in precipitated porcine sperm extracts and also correctly identified the recombinant protein (Fig. 2). The gpd/oscillin immunoreactive band in sperm extracts showed a slightly higher molecular weight than the recombinant product, suggesting that in porcine sperm gpd/oscillin may be posttranslationally modified. The presence of truncated c-kit in our extracts was investigated by Western blotting; truncated c-kit was absent from our preparations, although it was detected in whole sperm (not shown). Together, these data show that the Ca²⁺-releasing activity of our extracts was precipitated by 50% saturating solutions of ammonium sulfate and that the antibody raised against the recombinant hamster molecule correctly identified gpd/oscillin in porcine sperm extracts; this antibody was used throughout the present investigation.

IEF of Porcine Cytosolic Sperm Extracts

Sperm cytosolic fractions were first subjected to IEF using a pH 3–10 gradient (Fig. 3A). Fractions were collected and

pooled, and the resulting fractions (1–6) were tested for Ca²⁺-releasing activity. Fraction 3, which contained proteins migrating between pH 6 and 7, exhibited maximal Ca²⁺-releasing activity (Fig. 3C). Other fractions contained no activity or considerable less Ca²⁺-releasing activity (Fig. 3C). Western blot analysis of all fractions revealed maximal accumulation of immunoreactive gpd/oscillin in fraction 3 (Fig. 3B).

To further isolate/purify the Ca²⁺ activity in sperm extracts, fraction 3 from single IEF was exposed to a second round of IEF using ampholytes with a pH range of 5–8. Fractions were collected, grouped within specific pH ranges, and tested for [Ca²⁺]ᵢ oscillation-inducing activity. Fractions with maximal Ca²⁺-releasing activity were in the pH range of 6.5–7.0 (Fig. 4C) and immunoreactive gpd/oscillin was not detected in these fractions (Fig. 4B). Conversely, fraction 1, which had enhanced amounts of gpd/oscillin, showed no Ca²⁺-releasing activity (Fig. 4C). Despite that maximal Ca²⁺-releasing activity was observed in fractions 2 and 3, these fractions did not exhibit activity enrichment when compared to the original pellet fraction as assessed by the threshold protein concentrations required to trigger oscillations (data not shown). Moreover, Coomassie blue staining revealed that several proteins remained in the active fractions (not shown). The latter, coupled to less than optimal protein recovery, may limit the use of double IEF as a step in the scheme to purify/isolate the active protein(s) in porcine sperm cytosolic extracts. However, double IEF was useful to separate Ca²⁺-releasing activity from the presence of immunoreactive gpd/oscillin, suggesting that a different protein(s) may be the component responsible for Ca²⁺ release in porcine sperm extracts.

IEF and Superose 12 FPLC Chromatography of Porcine Cytosolic Sperm Extracts

Active fractions from sperm cytosolic extracts collected after single IEF were further fractionated by size exclusion chromatography to estimate the molecular weight of their proteins and to test the possibility that the lack of Ca²⁺ activity associated with the presence of immunoreactive gpd/oscillin after double IEF was due to degradation/denaturation of the native protein. The assumption was that if gpd/oscillin was modified by IEF, it may elute with a molecular weight(s) different from its estimated 200 kDa in native state (Parrington *et al.,* 1996; Wolosker *et al.,* 1998). The single IEF run for this particular experiment was carried out using 2% ampholytes with wide (pH 3–10, 17%) and narrow (pH 5–8, 83%) pH ranges; the Superose 12 column was calibrated with proteins of known molecular weights to estimate the size of the proteins in the extracts (Fig. 5C). Fraction 4 from Superose 12 showed maximal Ca²⁺-releasing activity (Fig. 5B) and eluted with volumes corresponding to molecular weight markers ranging from 29 to 68 kDa (Fig. 5C). SDS–PAGE analysis of the active fraction revealed several proteins of ≈40–150 kDa molecu-
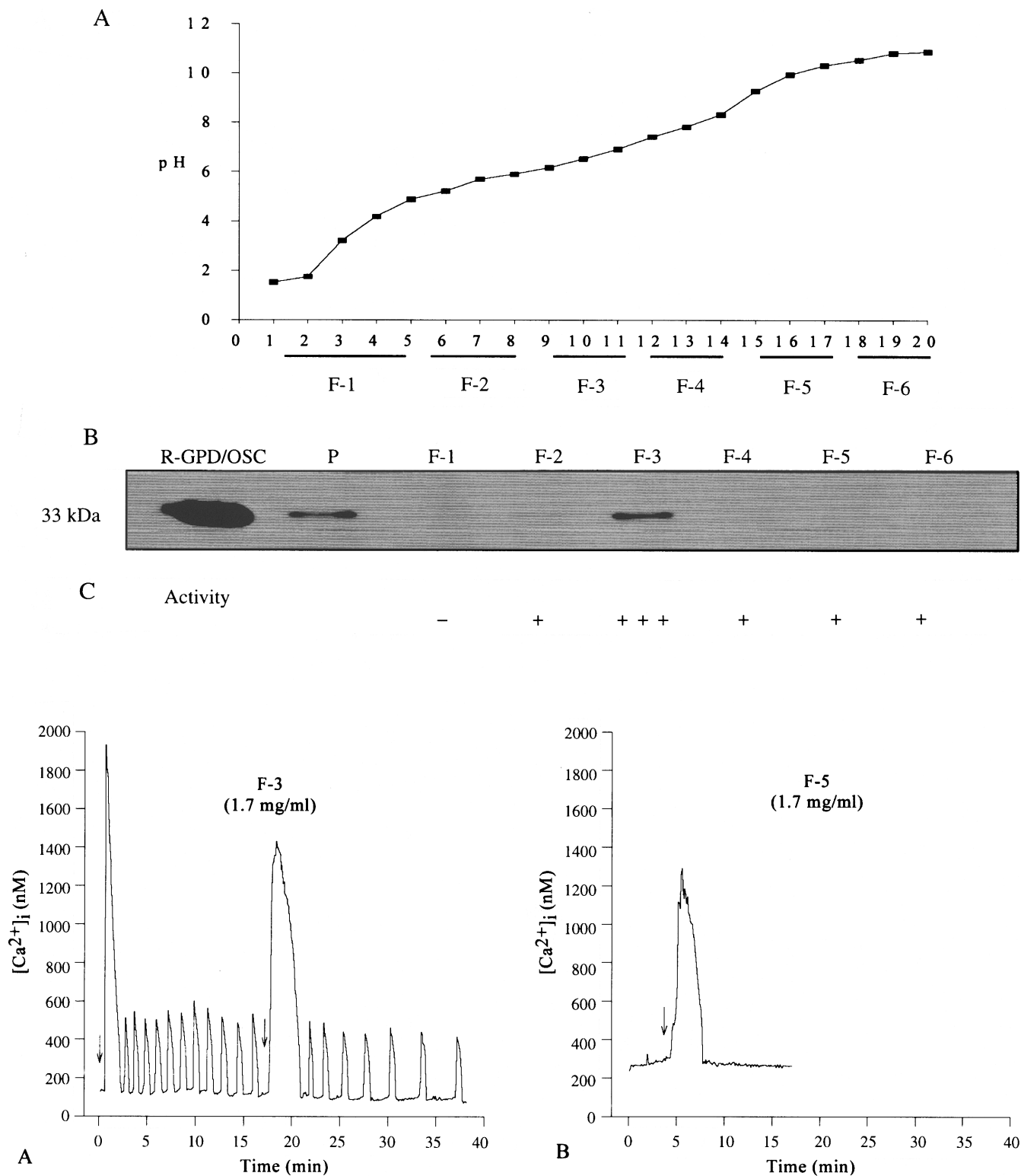
FIG. 3. IEF fractionation of porcine sperm extracts. Single IEF was conducted using ampholytes with a pH range of 3–10 (A); fractions were then pooled and the resulting fractions were used to test the presence of immunoreactive gpd/oscillin (B), or Ca^{2+} activity (C). Immunoreactive gpd/oscillin was detected in fraction 3, which contained maximal Ca^{2+} activity (+++), and faintly in fraction 2, which had minor activity (+). The pellet (P), which was the starting material before fractionation, and recombinant gpd/oscillin (R-gpd/osc) also showed an immunoreactive band. Other fractions showed some activity (+) or no activity (−) and contained no immunoreactive gpd/oscillin. Time of injection is denoted by an arrow and the injection pipet was removed immediately after injection; each arrow indicates injection into a different/new egg. The IEF procedure was repeated three different times and each fraction was tested for Ca^{2+} activity by injecting at least two eggs each time. Ca^{2+} activity of each fraction was highly consistent between experiments.

A

First running IEF Fractions

F-1   F-2   F-3   F-4   F-5   F-6

Second Running IEF Fractions

B

R-gpd/osc   F′-1   F′-2   F′-3   F′-4

33 kDa

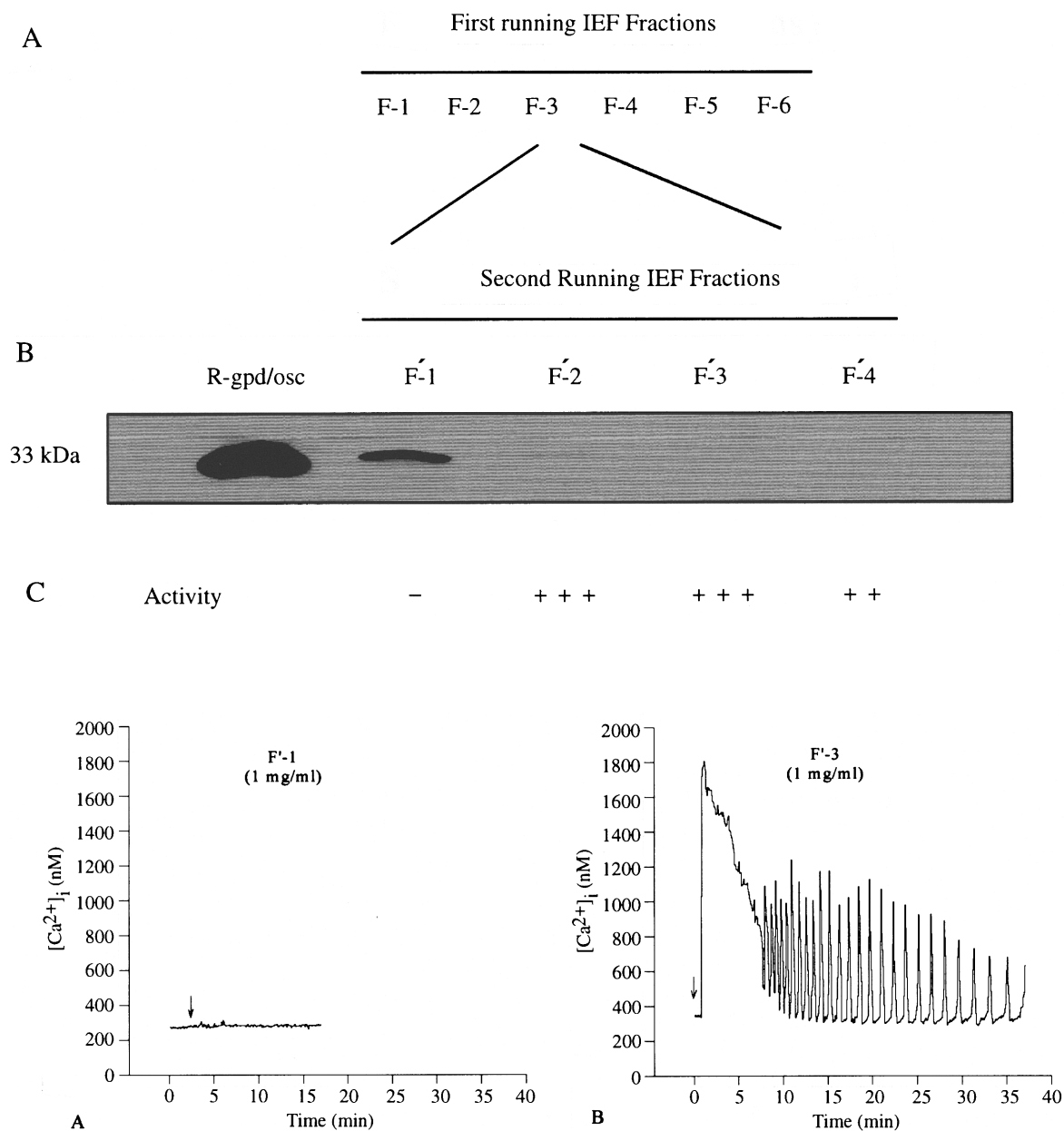C   Activity   −   + + +   + + +   + +

FIG. 4. Double IEF fractionation of porcine sperm extracts. After single IEF (A), fraction 3, which was the active fraction, underwent a second round of IEF using ampholytes with pH range of 5–8. Fractions were pooled and tested for immunoreactive oscillin (B) and Ca^{2+} activity (C). Immunoreactive gpd/oscillin accumulated in fraction 1 (pH range of 4.5–6.2) and this fraction had no Ca^{2+} activity (−). Maximal activity (+++) was detected in fractions 2 (pH 6.3–6.6) and 3 (pH 6.7–6.9) which showed no immunoreactive gpd/oscillin (faint band in fraction 2). Double IEF was carried out three separate times and at least two eggs per fraction were injected each time to test for Ca^{2+} activity. Ca^{2+} activity of each fraction was highly consistent between experiments.

lar weight (not shown). This fraction did not contain immunoreactive gpd/oscillin (Fig. 5A). Gpd/oscillin eluted mostly in fraction 2. The estimated molecular weight of proteins eluting in this fraction was 200 kDa (Fig. 5C), which is in agreement with the predicted size of native gpd/oscillin. In addition, when unprocessed crude sperm extracts were directly poured onto the same Superose 12 column, gpd/oscillin eluted with the same volume observed after IEF fractionation (not shown). Together, these data show that porcine sperm fractions with maximal Ca^{2+}-

A

IEF Fractions

F-1   F-2   F-3

Superose 12 Fractions

R-gpd/osc	P	F'-2	F'-1	F'-2	F'-3	F'-4	F'-5

33 kDa

B

Activity   −   −   −   +++   +

BSA (5 mg/ml)

F'-2 (2 mg/ml)

F'-4 (2.5 mg/ml)

C

releasing activity obtained after IEF processing do not contain immunoreactive gpd/oscillin despite that gpd/oscillin appears to conserve its native state.

Hydroxyapatite Chromatography of Porcine Cytosolic Sperm Extracts

Hydroxyapatite fractionation has been utilized to characterize the active components of hamster cytosolic sperm extracts (Parrington *et al.,* 1996). Thus, this column was utilized to fractionate our porcine sperm fractions. Proteins were eluted from the column by increasing the phosphate concentration in the buffer in a stepwise manner (Fig. 6A). Eluted proteins were collected, concentrated, and tested for Ca^{2+} activity. Ca^{2+}-releasing activity was observed in several fractions. Fractions 3 and 4, which contained maximal activity, eluted at 185 and 244 mM potassium phosphate, respectively. Fraction 1, which eluted at 88 mM potassium phosphate, had no Ca^{2+}-releasing activity and contained most of the immunoreactive gpd/oscillin (Figs. 6B and 6C). These data demonstrate that porcine cytosolic sperm extracts have the capacity to induce $[Ca^{2+}]_i$ oscillations in the absence of immunoreactive gpd/oscillin. Coomassie blue staining of the active fractions revealed numerous proteins (Fig. 6D), demonstrating the complexity of the protein profile in crude sperm fractions. These results suggest that the isolation/purification of the active component(s) in our extracts will require a thorough and extensive combination of fractionation procedures.

DISCUSSION

The present work confirms our previous findings that porcine cytosolic sperm extracts induce $[Ca^{2+}]_i$ oscillations when injected into mouse eggs and demonstrates that this activity can be fractionated by different chromatographic procedures. IEF fractionation and IEF coupled to Superose 12 predicts, for the active component(s), a pI of 6.5–7.0 and a relative molecular weight of 29–68 kDa. These data suggest a novel Ca^{2+}-releasing component(s) in porcine sperm extracts.

If the sperm's Ca^{2+}-releasing molecule is solely contained within the sperm cell cytosol, injection of sperm extract that is equivalent to a single sperm should initiate all events of fertilization. Previous studies have indicated that injections of extracts from sperm of several species represented 1–20 sperm equivalents; these amounts induced $[Ca^{2+}]_i$ oscillations and triggered activation (Stice and Robl, 1990; Swann, 1993; Stricker, 1997). In our study, sperm equivalents were calculated based on the total number of sperm per collection and the total amount of protein obtained after sonication, ultracentrifugation, and concentration of the extracts. An average boar ejaculate contained 4×10^{10} sperm and produced 80 mg of protein. Thus, each picogram of sperm extract protein contained 0.5 sperm equivalents. Since activity was assessed by injecting protein concentrations at 1 mg/ml (1 pg/pl) and injection volumes ranged from 5 to 10 pl, we estimate that each injection represented 2.5–5 sperm equivalents. Moreover, presently, using semipurified fractions, we are able to consistently initiate persistent $[Ca^{2+}]_i$ oscillations utilizing protein concentrations as low as 0.1 pg/pl. Therefore, although our calculations may not be exact, we assume complete extraction of the active component from every sperm, exclusive contribution of sperm cytosolic proteins, and do not take into account loss of protein activity that may occur during processing; they clearly demonstrate that the amounts of injected sperm extracts are within physiological values. Furthermore, the fact that several proteins are still present in our purified fractions reveals that porcine sperm extracts contain a powerful Ca^{2+}-releasing agent.

Several molecules have been suggested to represent the Ca^{2+} release active component of the sperm including Ca^{2+} (Jaffe, 1980), InsP3 (Iwasa *et al.,* 1990; Tosti *et al.,* 1993), and oscillin (Parrington *et al.,* 1996). Ca^{2+} and InsP3 are certainly participants in the generation of Ca^{2+} responses at fertilization, but single release of these compounds into eggs, which is likely what occurs during sperm penetration, has been shown to be unable to sustain long-lasting $[Ca^{2+}]_i$ oscillations or to sensitize the egg's Ca^{2+} release mechanisms (Igusa and Miyazaki, 1983; Miyazaki, 1988; Fissore and Robl, 1992; and 1993). Oscillin, which was later shown to be gpd (Wolosker *et al.,* 1998), has interesting features to support its possible role as the sperm's active component. Most importantly, it is located precisely at the site of sperm–egg fusion, it is cytosolic, and it is well conserved among species (Parrington *et al.,* 1997; Swann and Lai,

FIG. 5. Single IEF of porcine sperm extracts coupled with Superose 12 fractionation. After single IEF (see Results), fraction 2 (pH 6.5–7.5), whose pH range was similar to those of the active fractions after double IEF, was poured onto a Superose 12 column (A) that was calibrated with proteins of known molecular weight (C). These fractions were pooled and tested for immunoreactive gpd/oscillin (A) and activity (B). Despite enrichment of gpd/oscillin in fraction 2 (estimated molecular weight of 200 kDa), this fraction showed no Ca^{2+} activity. Fraction 4, which exhibited maximal activity, did not contain immunoreactive gpd/oscillin. Bovine serum albumen (BSA) was injected as a negative control. Time of injection is denoted by an arrow and the injection pipet was removed immediately after injection; each arrow indicates injection into a different/new egg. P was the pellet from which fractionation took place and R-gpd/osc was as for other figures. F-2 is the IEF fraction that was poured onto the Superose 12 column. IEF coupled with Superose 12 experiments was repeated twice and Ca^{2+} activity of each fraction was examined in at least two eggs each time. Ca^{2+} activity of fractions was consistent between replicates. IEF fractions 1 (pH 2.0–6.4) and 3 (pH 8.0–10.1) were not processed further.
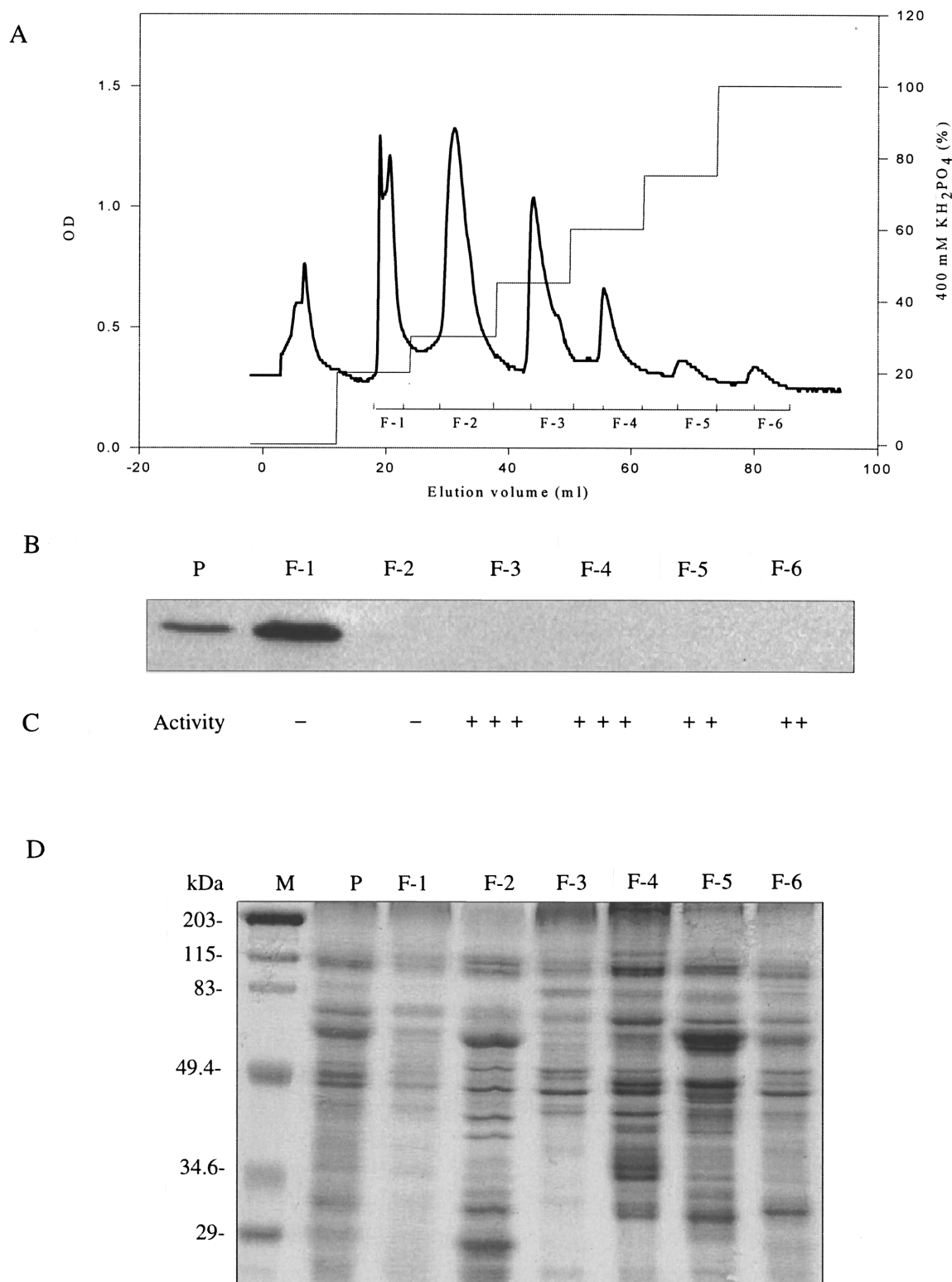
FIG. 6. Hydroxyapatite fractionation of porcine sperm extracts. Proteins were poured onto a hydroxyapatite column and eluted by increasing, in a step manner, the molarity of potassium phosphate in the buffer; the final step contained 400 mM potassium phosphate (A). Fractions were pooled, concentrated, and tested for the presence of immunoreactive gpd/oscillin (B) and Ca^{2+} activity (C). Fractions 3 and 4, which lacked immunoreactive gpd/oscillin, had maximal activity. Coomassie blue staining of fractions from the hydroxyapatite column (D); 30 μg of total protein was loaded per lane. P indicates pellet. Hydroxyapatite fractionation was carried three separate times and at least two eggs per fraction were injected each time to test Ca^{2+} activity. Ca^{2+} activity of each fraction was highly consistent between experiments.

1997; Fissore *et al.,* 1998). The role of gpd/oscillin, however, has been questioned recently when it was demonstrated that gpd/oscillin is expressed in several tissues, and that neither the recombinant form of the protein nor the native protein affinity purified from hamster sperm have Ca^{2+}-releasing activity (Wolosker *et al.,* 1998). This last paper, however, did not demonstrate if the fractions depleted of gpd/oscillin retained Ca^{2+}-releasing activity.

In a previous study we demonstrated that the active Ca^{2+} release component of porcine sperm extracts contains a protein moiety as its activity was eliminated by heat treatment or by exposure to proteinase K, a broad spectrum protease (Wu, *et al.,* 1997). The present study shows that the Ca^{2+}-releasing activity of these extracts can be fractionated and that gpd/oscillin may not be the active component. Fractions that contained no immunoreactive gpd/oscillin exhibit maximal Ca^{2+}-releasing activity and, conversely, fractions with enhanced levels of immunoreactive gpd/oscillin show decreased or no Ca^{2+}-releasing activity. This finding may indicate that sperm extracts from different species possess different active compounds, or that gpd/oscillin was misidentified in our fractions or, alternatively, that a different protein(s) is responsible for the Ca^{2+}-releasing activity in our fractions and other sperm extracts including the hamster. Regarding the first point, if the release of a sperm cytosolic molecule(s) represents the mechanism by which the sperm initiates $[Ca^{2+}]_i$ oscillations in mammalian eggs, it is likely that the putative molecule(s) would be highly conserved across mammalian sperm. Second, the specificity of our antibody was demonstrated by using a different anti-gpd/oscillin polyclonal antibody that showed a band of identical molecular weight to the one detected by our antibody. Thus, we favor the possibility that the Ca^{2+}-release-activating component(s) present in mammalian sperm extracts has not yet been identified.

Toward this end, fractionation of porcine sperm extracts by double IEF predicted a p*I* of 6.5–7.0 for the active component(s). IEF separation was very useful to segregate active fractions from the presence of gpd/oscillin; however, it resulted in very poor protein recovery. Moreover, purified IEF fractions did not exhibit enhanced $[Ca^{2+}]_i$ oscillation-inducing activity. The latter may be due to the disruption of natural complexes, or to the fact that more than one protein may actively participate in the generation of $[Ca^{2+}]_i$ oscillations and as they migrate to their p*I*s and separate to different fractions the specific activity of each fraction decreases, or that a proportion of some proteins may be permanently inactivated at their p*I*. Which of these possibilities or if a combination of them is occurring in our system is not known but it will limit the usefulness of this technique in the purification scheme. IEF coupled with Superose 12 column predicted a relative molecular weight of the active component of 29–68 kDa. This suggested molecular weight may not be the molecular weight at which the active factor operates physiologically as IEF may disrupt complexes or associations with other proteins and

these complexes may represent the correct native active state. IEF coupled with a Superose 12 did not appear to grossly modify gpd/oscillin which eluted from the Superose 12 column with its predicted native molecular weight of 200 kDa (Parrington *et al.,* 1996).

The segregation of Ca^{2+}-releasing activity from the presence of immunoreactive gpd/oscillin was also observed after hydroxyapatite fractionation. Our hydroxyapatite chromatography results differ from those of Parrington *et al.* (1996) in other aspects. In our study the most active fractions eluted at potassium phosphate concentrations in excess of 185 mM and gpd/oscillin eluted at 88 mM potassium phosphate. In contrast, in the aforementioned report, the active fractions eluted at around 18–24 mM potassium phosphate, which was the molarity at which gpd/oscillin also eluted. Whether these differences can be attributed to the buffer used to wash and elute the column, in our case increasing concentrations of potassium phosphate were used and in the other report 100 mM potassium chloride plus increasing concentrations of potassium phosphate were employed or to another technical factor is not known. A similarity among our reports was the finding that more than one hydroxyapatite fraction contained Ca^{2+}-releasing activity. Whether this represents two different proteins, or the same protein complexed to other proteins, or different isoforms of the same protein is under investigation.

In conclusion, our results demonstrate that the $[Ca^{2+}]_i$ oscillation-inducing activity of sperm extracts can be fractionated, that this activity in porcine fractions does not correlate with the presence of gpd/oscillin, and that the protein complexity of these extracts will require multiple purification steps to isolate the putative active protein(s). However, it is more than evident that sperm extracts contain a very powerful $[Ca^{2+}]_i$ oscillation-inducing component(s) and its isolation/purification may contribute to the elucidation of the mechanism by which the sperm initiates egg activation and embryonic development.

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REFERENCES

Almeida, E. A. C., Huovila, A-P., J., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., Myles, D. G., and White, J. M. (1995). Mouse egg integrin α6β1 functions as a sperm receptor. *Cell* **81,** 1095–1104.

Bronson, R. A., Gailit, J., Bronson, S., and Oula, L. (1995). Echistatin, a desintegrin, inhibits sperm–oolemal adhesion but not oocyte penetration. *Fertil. Steril.* **64,** 414–420.

Dale, B., DeFelice, L., J., and Ehrenstein, G. (1985). Injection of a soluble sperm extract into sea urchin eggs triggers the cortical reaction. *Experientia* **41,** 1068–1070.

Dozortsev, D., Rybouchkin, A., De Sutter, P., Qian, C., and Dhont, M. (1995). Human oocyte activation following intracytoplasmic injection: The role of the sperm cell. *Hum. Reprod.* **10,** 403–407.

Dupont, G., McGuinness, O. M., Johnson, M. H., Berridge, M. J., and Borghese, F. (1996). Phospholipase C in mouse oocytes: characterization of β and γ isoforms and their possible involvement in sperm-induced Ca^{2+} spiking. *Biochem. J.* **316,** 583–591.

Evans, J. P., Schultz, R. M., and Kopf, G. S. (1995). Identification and localization of integrin subunits in oocytes and eggs in the mouse. *Mol. Reprod. Dev.* **20,** 211–220.

Fissore, R. A., and Robl, J. M. (1992). Intracellular Ca^{2+} response of rabbit oocytes to electrical stimulation. *Mol. Reprod. Dev.* **32,** 9–16.

Fissore, R. A., and Robl, J. M. (1993). Sperm, inositol trisphosphate, and thimerosal-induced intracellular Ca^{2+} elevations in rabbit eggs. *Dev. Biol.* **159,** 122–130.

Fissore, R. A., Gordo, A. C., and Wu, H. (1998). Activation of development in mammals: Is there a role for a sperm cytosolic factor? *Theriogenology* **49,** 43–52.

Fusi, F. M., Vignali, M., Gailit, J., and Bronson, R. A. (1993). Mammalian oocytes exhibit specific recognition of RGD (Arg-Gly-Asp) tripeptide and express oolemal integrins. *Mol. Reprod. Dev.* **36,** 212–219.

Galione, A., Jones, K. T., Lai, A. F., and Swann, K. (1997). A cytosolic sperm protein factor mobilizes Ca^{2+} from intracellular stores by activating multiple Ca^{2+} release mechanisms independently of low molecular weight messengers. *J. Biol. Chem.* **272,** 28901–28905.

Grynkiewicz, G., Poenie, M., and Tsien, T. Y. (1985). A new generation of calcium indicators with greatly enhanced fluorescent properties. *J. Biol. Chem.* **260,** 3440–3450.

He, C. L., Damiani, P., Parys, J. B., and Fissore, R. A. (1997). Calcium, calcium release receptors, and meiotic resumption in bovine oocytes. *Biol. Reprod.* **57,** 1245–1255.

Homa, S. T., and Swann, K. (1994). A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. *Hum. Reprod.* **9,** 2356–2361.

Igusa, Y., and Miyazaki, S. (1983). Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *J. Physiol.* **340,** 611–632.

Iwao, Y., and Fujimura, T. (1996). Activation of *Xenopus* eggs by RGD-containing peptides accompanied by intracellular Ca^{2+} release. *Dev. Biol.* **177,** 558–567.

Iwasa, K. H., Ehrenstein, G., DeFelice, L. J., and Russell, J. T. (1990). High concentrations of inositol 1,4,5-trisphosphate in sea urchin sperm. *Biochem. Biophys. Res. Commun.* **172,** 932–938.

Kimura, Y., and Yanagimachi, R. (1995). Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* **121,** 2397–2405.

Jaffe, L. (1980). Calcium explosions as triggers of development. *Ann. NY Acad. Sci.* **339,** 86–101.

Kline, D., and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149,** 80–89.

Miyazaki, S. (1988). Fertilization potential and calcium transient in mammalian eggs. *Dev. Growth Differ.* **30,** 603–610.

Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., and Igusa, Y. (1986). Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev. Biol.* **118,** 259–267.

Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role for the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev. Biol.* **118,** 259–267.

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y., and Miyazaki, S. (1997). Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with spermatozoon. *Mol. Hum. Reprod.* **3,** 1087–1093.

Palermo, G. D., Joris, H., Devroey, P., and van Sterteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* **340,** 17–18.

Palermo, G. D., Avrech, O. M., Colombero, L. T., Wu, H., Wolny, Y. M., Fissore, R. A., and Rosenwaks, Z. (1997). Human sperm cytosolic factor triggers Ca^{2+} oscillations and overcomes activation failure of mammalian oocytes. *Mol. Hum. Reprod.* **3,** 364–374.

Parrington, J., Swann, K., Shevchenko, V. I., Sesay, A. K., and Lai, F. A. (1996). Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* **379,** 364–368.

Parrish, J. J., Susko-Parrish, J., Winer, M. A., and First, N. L. (1988). Capacitation of bovine sperm by heparin. *Biol. Reprod.* **38,** 1171–1180.

Poenie, M. (1990). Alteration of intracellular fura-2 fluorescence by viscosity: A simple correction. *Cell Calcium* **11,** 85–91.

Schultz, R. M., and Kopf, G. S. (1995). Molecular basis of mammalian egg activation. *Curr. Top. Dev. Biol.* **30,** 21–62.

Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R., and Rossi, P. (1997). Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development* **124,** 2267–2274.

Snell, W. J., and White, J. M. (1996). The molecules of fertilization. *Cell.* **85,** 629–637.

Souza, M., Mendoza, C., Barros, A., and Tesarik, J. (1996). Calcium responses of human oocytes after intracytoplasmic sperm injection of leukocytes, spermatocytes and round spermatids. *Mol. Hum. Reprod.* **2,** 853–857.

Stice, S. L., and Robl, J. M. (1990). Activation of mammalian oocytes by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.* **25,** 272–280.

Striker, S. (1997). Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized eggs of a marine worm. *Dev. Biol.* **186,** 185–201.

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110,** 1295–1302.

Swann, K. (1993). The soluble sperm oscillogen hypothesis. *Zygote* **1,** 273–276.

Swann, K., and Lai, F. A. (1997). A novel signaling mechanisms for generation Ca^{2+} oscillations at fertilization in mammals. *BioEssays* **19,** 371–378.

Tesarik, J., Souza, M., and Testart, J. (1994). Human oocyte activation after intracytoplasmic sperm injection. *Hum. Reprod.* **9,** 511–518.

Tosti, E., Palumbo, A., and Dale, B. (1993). Inositol 1,4,5-trisphosphate in human and ascidian spermatozoa. *Mol. Reprod. Dev.* **35,** 52–56.

Williams, C. J., Mehlmann, L. M., Jaffe, L. A., Kopf, G. S., and Schultz, R. M. (1998). Evidence that G_q family proteins do not

function in mouse egg activation at fertilization. *Dev. Biol.* **198,** 116–127.

Williams, C. J., Schultz, R. M., and Kopf, G. S. (1992). Role of G proteins in mouse egg activation: Stimulatory effects of acetylcholine on the ZP2 to ZP2f conversion and pronuclear formation in eggs expressing a functional m1 muscarinic receptor. *Dev. Biol.* **151,** 288–296.

Whitaker, M. J., and Patel, R. (1990). Calcium and cell cycle control. *Development* **108,** 525–542.

Whitaker, M. J., and Swann, K. (1993). Lighting the fuse at fertilization. *Development* **117,** 1–12.

Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A. M., Fralich, T. J., Schnaar, R. L., and Snyder, S. H. (1998). Molecularly cloned mammalian glucosamine-6-phosphate deaminase localizes to trasnopoting epithelium and lacks oscillin activity. *FASEB J.* **12,** 91–99.

Wu, H., He, C. L., and Fissore, R. A. (1997). Injection of a porcine sperm factor triggers calcium oscillations in mouse oocytes and bovine eggs. *Mol. Reprod. Dev.* **46,** 176–189.

Wu, H., He, C. L., and Fissore, R. A. (1998). Injection of a porcine sperm factor induces activation in mouse eggs. *Mol. Reprod. Dev.* **49,** 37–47.