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Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx

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Hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate is thought to be intimately involved in agonist-induced changes in intracellular Ca^{2+} levels. Recently we have shown that human preovulatory follicular fluid, which induces exocytosis in human sperm, can stimulate a rapid, transient increase in sperm cytosolic [Ca^{2+}] [Thomas & Meizel (1988) Gamete Res. **20**, 397–411]. We report here that both a Sephadex G-75 column fraction, derived from follicular fluid, and progesterone (a component of both the G-75 fraction and whole follicular fluid) stimulate rapid hydrolysis of PtdIns(4,5)P_2 and PtdIns4P in human sperm. We also report that progesterone stimulates a rapid influx of Ca^{2+} in human sperm. Human spermatozoa were labelled for 24 h with *myo*-[^{3}H]inositol and then treated with either the G-75 fraction or progesterone. A 30–65% loss of label was detected in PtdIns(4,5)P_2 and PtdIns4P within 15 s of stimulus addition; no changes were observed in PtdIns during 2 min of treatment. The loss of label from both lipids was accompanied by an increase in water-soluble inositol phosphates. Production of both InsP_3 and InsP_2 was seen within 10 s; however, InsP_3 was rapidly removed and had reached control levels by 1 min. Similarly, formation of InsP_2 reached a peak by 30 s and then began a decline accompanied by a corresponding increase in Ins$P$. No increases in InsP_4 were seen in sperm treated in this fashion. Stimulated hydrolysis of the phosphoinositides and release of inositol phosphates were both blocked by the Ca^{2+} antagonist La^{3+}. Likewise, the progesterone-induced increase in intracellular Ca^{2+} was inhibited by La^{3+}, and phosphoinositide hydrolysis stimulated by this hormone was dependent upon the presence of extracellular Ca^{2+}.

INTRODUCTION

In order to fertilize oocytes, spermatozoa must first undergo an exocytotic event known as the acrosome reaction [1,2]. The acrosome reaction is thought to be stimulated by components of the vestments surrounding the ovulated oocyte [2,3]. The oocyte of placental mammals, both before and after ovulation, is surrounded by a mass of cells embedded in an extracellular matrix, known collectively as the cumulus oophorus. In the ovary prior to ovulation, the secretions of the cumulus cells, and of the closely related granulosa cells, accumulate within the antrum of the follicle. It has been demonstrated that supernatants from cultured human cumulus [4,5] and granulosa cells [5], and the fluid obtained from pre-ovulatory human ovarian follicles [4,6,7] can stimulate the acrosome reaction in capacitated human sperm *in vitro*.

Following fractionation of preovulatory ovarian follicular fluid using Sephadex G-75 column chromatography [6,7], acrosome reaction-inducing activity was tentatively ascribed to a protein or glycoprotein of apparent M_r of approx. 50000. Using fura-2, we were able to demonstrate that this G-75 fraction stimulated a rapid, transient increase in intracellular [Ca^{2+}] in human sperm which seemed to be due to an influx of extracellular Ca^{2+} [8]. While the present work was in progress, however, other studies in this laboratory [9] determined that much of the acrosome reaction-inducing activity of follicular fluid could be explained by progesterone. Significantly, the presence of progesterone has since been demonstrated in two G-75 fractions assayed (S. Meizel, unpublished work).

Metabolism of the minor membrane phospholipid PtdIns(4,5)P_2 has been implicated in the regulation of Ca^{2+} mobilization in many tissues in response to a variety of agonists [10]. In spermatozoa, however, the relationship between PtdIns(4,5)P_2 hydrolysis and Ca^{2+} mobilization is uncertain. It seems clear that in spermatozoa the acrosome reaction is triggered by an influx of extracellular Ca^{2+} [8,11]. PtdIns(4,5)P_2 hydrolysis has generally been associated with the release of Ca^{2+} from intracellular stores [12]. However, it has been suggested that a metabolic product of PtdIns(4,5)P_2 hydrolysis, Ins(1,3,4,5)P_4, together with the immediate product of hydrolysis, Ins(1,4,5)P_3, might regulate Ca^{2+} influx through the plasma membrane [13,14]. Other work on lymphocytes and mast cells has even suggested that Ca^{2+} influx may be controlled by Ins(1,4,5)P_3 alone [15,16].

In the sea urchin, the morphologically equivalent structure to the cumulus oophorus is the egg jelly. Acrosome reaction-inducing activity of the egg jelly has

Abbreviations used: PPI, polyphosphoinositide; FSG, fucose sulphate glycoconjugate; G-75FR, fraction of human follicular fluid containing acrosome reaction-inducing activity; HEEDTA, *N*-hydroxy-EDTA; BSA, bovine serum albumin; TCA, trichloroacetic acid; VOCC, voltage-operated Ca^{2+} channel; SMOCC, second-messenger-operated Ca^{2+} channel; ROCC, receptor-operated Ca^{2+} channel.

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been shown to reside in a fucose sulphate glycoconjugate (FSG) [17]. Recently, Domino & Garbers [18] reported that inositol phosphates were increased in sea urchin spermatozoa when stimulated with FSG. Nevertheless, the generation of inositol phosphates was completely dependent upon the FSG-induced Ca^{2+} influx, and they observed no increase in Ins(1,3,4,5)P_4.

In mammalian sperm, the presence of the phosphoinositides and of the enzyme responsible for their cleavage has been established [19,20]. Likewise, loss of PtdIns [21] and the polyphosphoinositides (PPIs) [22,23] has been observed in spermatozoa undergoing acrosome reactions on treatment with the ionophore A23187, or spontaneously during incubation for several hours. Nevertheless, in mammalian sperm, turnover of the phosphoinositides in direct response to a physiological agonist has not been demonstrated. We therefore initiated these studies in order to establish the relationship, if any, between follicular fluid-induced Ca^{2+} influx and phosphoinositide metabolism in human sperm.

MATERIALS AND METHODS

Materials

The following were purchased: Percoll, bovine serum albumin (BSA) (cat. no. 7030), penicillin G, streptomycin sulphate, citric acid, Ada, HEEDTA, *myo*-inositol, progesterone, Sephadex G-75, phosphoinositides and phytic acid from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Hepes from Research Organics, Inc. (Cleveland, OH, U.S.A.); fura-2/AM from Molecular Probes (Junction City, OR, U.S.A.); UM2 ultrafiltration filters from Amicon Corp. (Lexington, MA, U.S.A.); AG 50W-X8 (100–200 mesh, H^+ form) from Bio-Rad Labs. (Richmond, CA, U.S.A.); Partisphere SAX column (10 μm particle size; 4.6 mm × 25 cm) from Whatman (Clifton, NJ, U.S.A.); t.l.c. plates from Analtech, Inc. (Newark, DE, U.S.A.); Ready-Safe and Ready-Value scintillation cocktails from Beckman (Fullerton, CA, U.S.A.); [^{3}H]Ins (15 Ci/mmol) from American Radiolabelled Chemicals Inc. (St. Louis, MO, U.S.A.); [^{3}H]Ins (83 and 99.5 Ci/mmol), [^{3}H]Ins1P, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 from Amersham Corp. (Arlington Heights, IL, U.S.A.). All common chemicals were purchased from Sigma Chemical Co., Mallinckrodt (Paris, KY, U.S.A.) or Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Incubation of spermatozoa

Human semen was obtained by masturbation from seven healthy donors. Populations of $\geqslant 95\%$ motile sperm uncontaminated by seminal plasma or other cell types were prepared using discontinuous Percoll gradients as described previously [8]. The sperm were finally suspended to a concentration of 6×10^6/ml in Medium A (Med. A), containing 117.5 mM-NaCl, 25 mM-$NaHCO_3$, 8.6 mM-KCl, 2.4 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, 0.3 mM-NaH_2PO_4, 19 mM-sodium lactate, 2.5 mM-sodium pyruvate, 0.05 mg of penicillin G/ml, 0.075 mg of streptomycin sulphate/ml and 26 mg of BSA/ml. Sperm were then incubated (in 0.8 ml aliquots) for 24 h at 37 °C in a humid atmosphere of CO_2/air (1:19) with 2.5–5 μCi of [^{3}H]Ins/ml (46–99.5 Ci/mmol).

Under these conditions, incorporation of [^{3}H]Ins into the phosphoinositides was within the following ranges: 2.8–7.6 fmol/10^7 cells in PtdIns(4,5)P_2, 1.4–3.9 fmol/10^7 cells in PtdIns4P and 1.3–4.3 fmol/10^7 cells in PtdIns.

Measurement of phosphoinositides

After the 24 h labelling period, aliquots of sperm were pooled into 0.25 vol. of Medium B (Med. B) (pH 7.55), containing 125 mM-NaCl, 10 mM-KCl, 2.5 mM-$CaCl_2$, 0.25 mM-$MgCl_2$, 19 mM-sodium lactate, 2.5 mM-sodium pyruvate, 20 mM-Hepes/NaOH and 3 mg of BSA/ml, which was supplemented with 10 mM-Ins. The cells were sedimented at 300 g for 10 min and the pellet was resuspended in 50 ml of Med. B (+10 mM-Ins) and subjected to a 40 min chase at 37 °C. The cell suspensions were then centrifuged at 300 g for 10 min and the pellet was resuspended to 10–12% of the original incubation volume [(3.5–4) × 10^7 sperm/ml] in Med. B. The cells were then placed in a water bath at 37 °C and 0.42 ml aliquots were treated with 0.08 ml of stimulus solution. Treated samples (0.5 ml) were extracted at the appropriate times with 1.88 ml of methanol/chloroform/conc. HCl (100:50:1, by vol.) on ice. To act as a carrier, 10 μl of a 10 mg/ml solution of crude phosphoinositides (in chloroform) was added to each extract. After extracting on ice for 30 min, the phases were separated by addition of 0.625 ml of chloroform and 0.625 ml of 2 M-KCl [24]. The lower chloroform layer was taken, dried down under N_2 and the residue was resuspended in a small volume of chloroform. The lipids were then separated on silica gel H plates impregnated with 1% potassium oxalate using a solvent system of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) [25]. Chromatograms were stained with iodine and the appropriate spots scraped from the plates. The spots were identified by their comigration with standard phospholipids. Each spot was hydrolysed at 180 °C with 0.2 ml of 70% $HClO_4$ for 45 min and then allowed to cool. To each hydrolysate was added 0.8 ml of water and 1 ml of 50% aqueous methanol, the sample was vortexed and then the silica was sedimented at 500 g for 2 min. Aliquots (1.5 ml) of the supernatants were added to 5 ml of Ready-Safe scintillant and radioactivity was counted (counting efficiency was approx. 35%).

Measurement of inositol phosphates

After the labelling period, the aliquots of sperm were pooled into 0.25 vol. of Med. B and then sedimented at 300 g for 10 min. The pellet was resuspended to 15 ml with Med. B and then allowed to 'rest' at 37 °C for 10 min before centrifuging again at 300 g for 10 min. The final pellet was resuspended to 10% of the original incubation volume ($\sim 4 \times 10^7$ cells/ml) in Med. B, this time supplemented with 10 mM-LiCl [26]. The cells were then incubated in 0.42 ml aliquots at 37 °C and treated with 0.08 ml of stimulus. After the appropriate time, the 0.5 ml samples were extracted with 0.5 ml of 20% (v/v) trichloroacetic acid (TCA) on ice. To each extract was added 10 μl of 25 mM 'hydrolysed' phytic acid (see below) as carrier. The samples were allowed to extract on ice for 30 min, the precipitates were sedimented at 9000 g for 2 min and the supernatants were washed with 10 vol. of water-saturated diethyl ether to remove the TCA. The lower aqueous phase from the wash was removed and neutralized with 5 M-NH_4OH and then evaporated to dryness using an evacuated centrifuge (Savant Instr. Inc., Farmingdale, NY, U.S.A.). The residues were stored dry at −80 °C until needed. To separate the inositol

phosphates, the residues were resuspended in 1.2 ml of 0.03 M-ATP and then, using a 1 ml loop, loaded on to a Whatman SAX-10 column. The column was eluted at a flow rate of 1.25 ml/min using a gradient of ammonium formate (pH 3.7 with phosphoric acid) similar to that described by Irvine *et al.* [27]. Fractions were collected every minute, and 1 ml aliquots from each were added to 10 ml of Ready-Value scintillant along with 2 ml of 50% aqueous methanol and the radioactivity was counted (counting efficiency was approx. 35%). The formate gradient was as follows: water for the first 5 min; a linear gradient from water to 1 M-ammonium formate over the next 20 min; a further ramp to 2 M-ammonium formate over the next 10 min; 2 M-ammonium formate was passed through the column for a further 15 min before returning to water over a period of 15 min. The column was eluted with water for another 15 min before applying the next sample.

Measurement of intracellular [Ca^{2+}]

Sperm suspensions (6×10^6/ml) in Med. A were loaded for 30 min with 1 μM-fura-2/AM and extracellular dye removed by centrifuging through 40% Percoll as described previously [8], except that sperm were not capacitated for these experiments. Additions were made to 1 ml aliquots of sperm suspensions ($\sim 6 \times 10^6$/ml in Med. B) in a stirred cuvette at 37 °C. Spectrofluorimetric assays were carried out as described by Thomas & Meizel [8] using a single wavelength of excitation (339 nm) whilst monitoring emission at 500 nm. Estimations of intracellular [Ca^{2+}] were made according to Pollock *et al.* [28].

Preparation of stimuli

The Sephadex G-75 fraction of follicular fluid (G-75FR) was prepared by chromatography as described by Siiteri *et al.* [7] except that the follicular fluid was not heat-treated, diluted or ultrafiltered before applying to the column. Fractions containing acrosome reaction-inducing activity were collected, pooled and then concentrated 4-fold (final protein concn. approx. 40 mg/ml) using an Amicon ultrafiltration device containing a UM2 (M_r 1000 cut-off) filter. For the Ca^{2+}-dependency of phosphoinositide breakdown, progesterone was dissolved in dimethyl sulphoxide to 200 μg/ml and then diluted to 7.14 μg/ml with Med. B supplemented with 23 mg of BSA/ml. This solution was further diluted to 6.25 μg of progesterone/ml with the appropriate chelator solution (see below) and added to labelled sperm (yielding a final progesterone concn. of 1 μg/ml). For the experiments with fura-2-loaded sperm, progesterone was dissolved to 0.1, 0.5 or 2 mg/ml in dimethyl sulphoxide and then diluted 50-fold with Med. B supplemented with 7 mg of BSA/ml. Aliquots (25 μl) of these solutions were then injected directly into the sperm suspension in the cuvette to give final progesterone concentrations of 0.05, 0.25 or 1 μg/ml respectively.

Hydrolysis of phytic acid

Phytic acid (inositol hexakisphosphate) was acid-hydrolysed to obtain a mixture of inositol phosphates as described by Wreggett & Irvine [29], except that desalting was carried out using AG 50W-X8 resin (H^+ form).

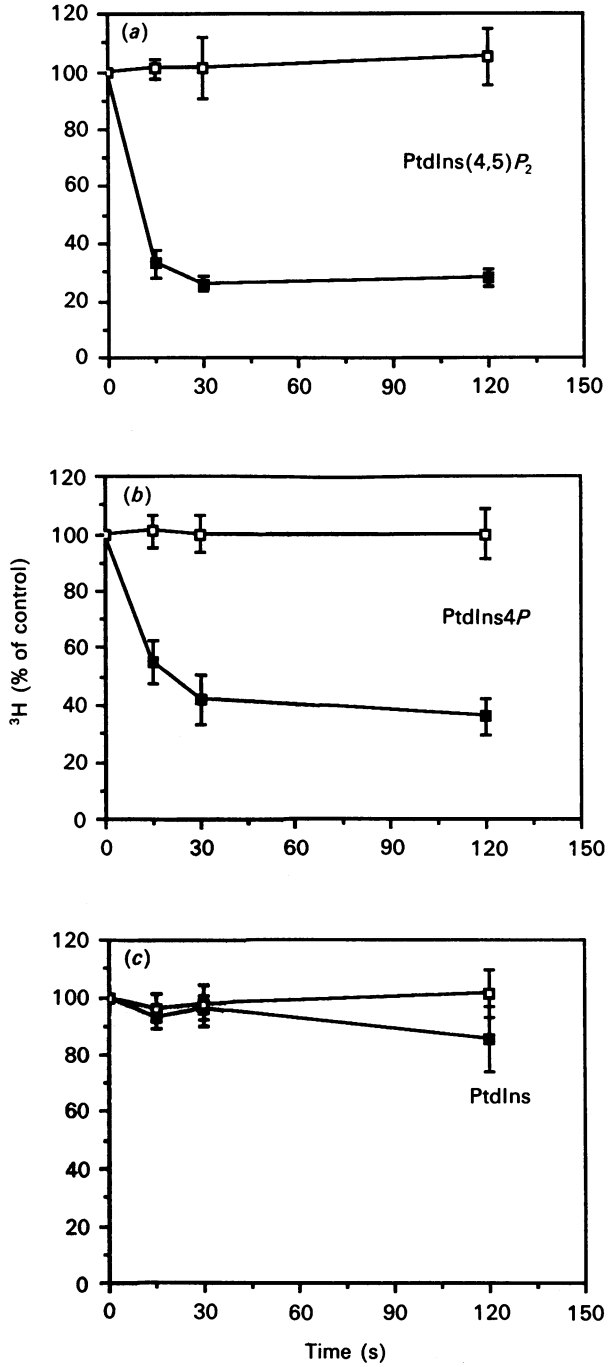

Fig. 1. Time course of the loss of phosphoinositides from human sperm stimulated with G-75FR

Human sperm ($\sim 4 \times 10^7$/ml) labelled with [^{3}H]Ins were treated with G-75FR at 37 °C for various times and then 0.5 ml samples were extracted with methanol/chloroform/conc. HCl. Phospholipids were separated by t.l.c. and individual lipids were scraped from the plates and radioactivity counted. Untreated control levels of the individual phosphoinositides (means ± S.E.M.) were: PtdIns(4,5)P_2, 1211 ± 395 d.p.m./sample; PtdIns4P, 528 ± 125 d.p.m./sample; PtdIns, 666 ± 257 d.p.m./sample. (*a*) PtdIns(4,5)P_2, (*b*) PtdIns4P, (*c*) PtdIns. ■, Samples treated with G-75FR alone; □, samples treated with G-75FR in the presence of 250 μM-La$(NO_3)_3$. Each point (mean ± S.E.M., $n = 3$) is expressed as a percentage of duplicate untreated controls. Motility assessed after the labelling period in these experiments was 80, 84 and 91%.

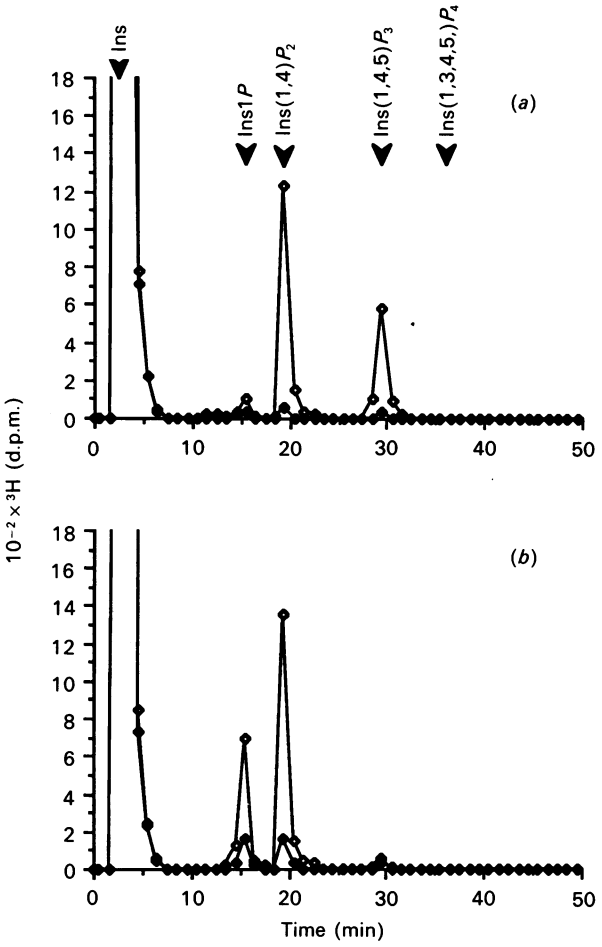

Fig. 2. Representative h.p.l.c. elution profiles of [^{3}H]inositol phosphates generated on stimulation of human sperm with G-75FR

Human sperm ($\sim 4 \times 10^7$/ml) prelabelled with [^{3}H]Ins were treated with G-75FR at 37 °C for various times and then 0.5 ml samples were extracted with an equal volume of 20% TCA. Extracts were prepared for h.p.l.c. and then separated as described in the Materials and methods section. (*a*) ◇, Extract of sperm treated with G-75FR for 10 s (ATP elution time 27.12 min); ◆, extract of untreated sperm incubated for 60 s (ATP elution time 27.11 min). (*b*) ◇, Extract of sperm treated with G-75FR for 60 s (ATP elution time 27.08 min); ◆, extract of sperm treated with G-75FR for 60 s in the presence of 250 μM-La^{3+} (ATP elution time 27.07 min).

Chelator solutions

Molar stock solutions of citrate/NaOH (pH 8.5), Ada/NaOH (pH 8.0) and HEEDTA/NaOH (pH 9.0) were made up, then diluted with water to give the following solutions: 250 mM-citrate, 250 mM-Ada, 500 mM-Ada, 1 M-Ada and 250 mM-HEEDTA. When added to Med. B (without BSA) at 50-fold dilutions, these chelators resulted in free [Ca^{2+}] of 398 μM (5 mM-citrate), 96 μM (5 mM-Ada), 45 μM (10 mM-Ada), 11 μM (20 mM-Ada) and 1.9 μM (5 mM-HEEDTA) as determined using a Radiometer F2112Ca Ca^{2+} selective electrode (Radiometer, Copenhagen, Denmark) which was standardized with Ca^{2+} buffers purchased from World Precision Instr. Inc. (New Haven, CT, U.S.A.). Addition of these chelator

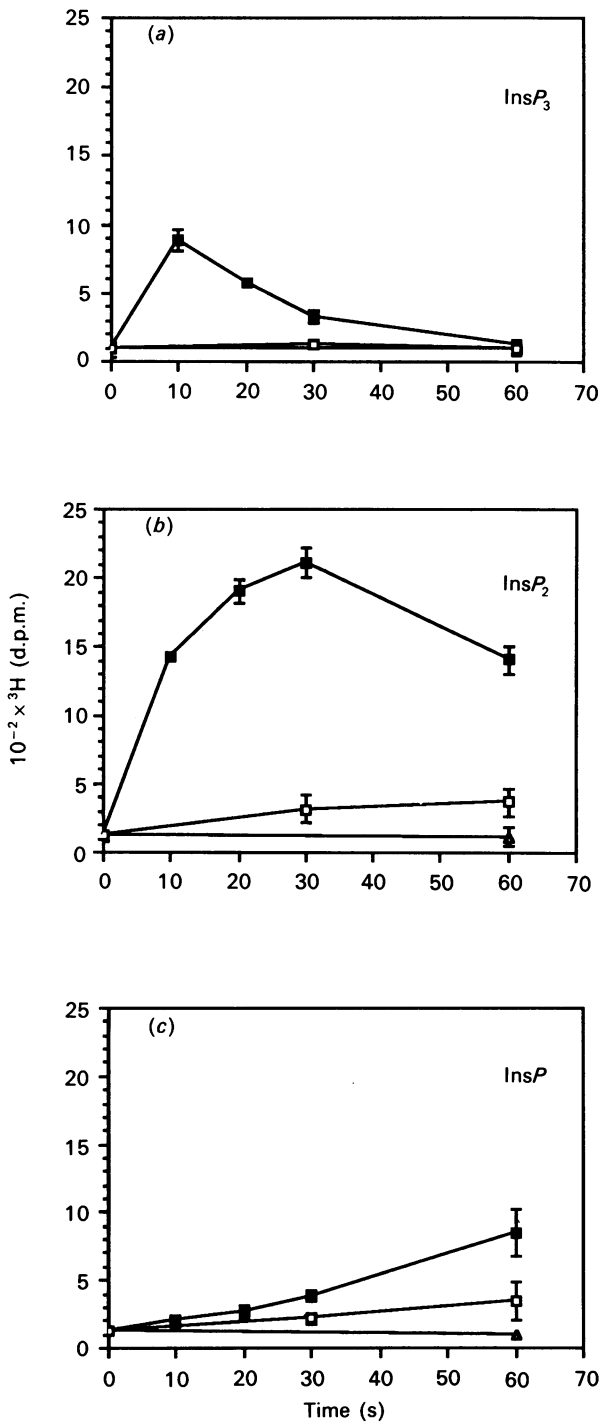

Fig. 3. Time course of the production of [^{3}H]inositol phosphates during stimulation of human sperm with G-75FR

Radiolabelled human sperm ($\sim 4 \times 10^7$/ml) were stimulated with G-75FR for various times at 37 °C and then 0.5 ml samples were extracted with TCA. Extracts were prepared for h.p.l.c. and then separated as described in the Materials and methods section. Fractions were collected every 1 min and 1 ml samples of each fraction were taken and radioactivity was counted. (*a*) InsP_3, (*b*) InsP_2, (*c*) Ins*P*. ■, Extracts of sperm treated with G-75FR; □, extracts of sperm treated with G-75FR in the presence of 250 μM-La^{3+}; △, extracts of untreated sperm. Each point is the mean ± S.E.M. derived from three individual experiments. Motility after labelling was 75, 82 and 88% in the three experiments.

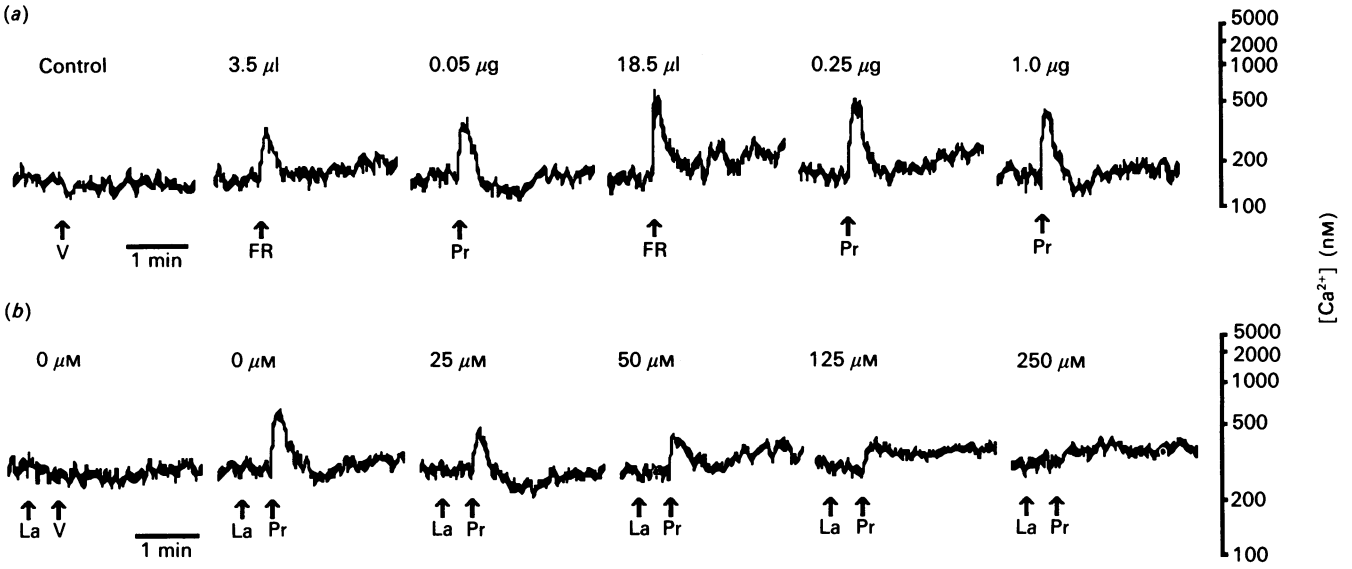

Fig. 4. Increase in intrasperm [Ca^{2+}] induced by progesterone and its inhibition by La^{3+}

Fura-2-loaded sperm were obtained as described in the Materials and methods section. Sperm suspensions (1 ml; $\sim 6 \times 10^6$/ml) were then treated with various stimuli at 37 °C in a stirred cuvette. Spectrofluorimetric assays were carried out at an excitation wavelength of 339 nm and an emission wavelength of 500 nm. (*a*) Sperm were treated with various amounts of progesterone, or different volumes of a G-75FR containing approx. 13.6 μg of progesterone/ml (see Fig. for amounts of progesterone or G-75FR). (*b*) Sperm were treated with 1 μg of progesterone after the addition of the indicated concentrations of $La(NO_3)_3$. V, dimethyl sulphoxide/BSA vehicle; FR, G-75FR; Pr, progesterone; La, $La(NO_3)_3$. Traces in (*a*) represent one of two similar experiments, and traces in (*b*) are representative of three similar experiments. In these five experiments motility at the end of the experiment ranged from 85 to 90%.

solutions to Med. B (±BSA) resulted in a pH change of no more than 0.04 pH unit.

RESULTS

G-75FR-induced hydrolysis of phosphoinositides

Treatment of sperm, which had been prelabelled with [^{3}H]Ins, with G-75FR led to a rapid loss of label from both PtdIns(4,5)P_2 (Fig. 1*a*) and PtdIns4P (Fig. 1*b*). The decreases in PtdIns(4,5)P_2 and PtdIns4P were about 70% and 60% respectively after 2 min. Essentially maximal loss of label had occurred by 30 s in these phospholipids; however, even after 2 min very little change was observed in PtdIns (Fig. 1*c*). Addition of 250 μM-$La(NO_3)_3$, a concentration which had previously been shown to completely block G-75FR-induced Ca^{2+} influx [8], totally prevented loss of label from these phospholipids (Figs. 1*a*–1*c*).

G-75FR-induced generation of inositol phosphates

The loss of label from PtdIns(4,5)P_2 and PtdIns4P was further characterized by analysis of the potential water-soluble products of phosphoinositide hydrolysis. Representative h.p.l.c. elution profiles of extracts of sperm treated with G-75FR are shown in Fig. 2. The elution times for a typical separation of ^{3}H-labelled standard inositol phosphates using this gradient are indicated on the Figure. In 19 sample runs (this includes the four samples shown in Fig. 2), using the same batch of ammonium formate, the mean elution time of ATP was 26.98 min (S.D. ±0.14 min). Five other samples were separated using a different batch of ammonium formate which gave slightly faster, but predictable, elution times (mean elution time of ATP, 25.65±0.02 min). The time course of the changes in inositol phosphates on stimulation of spermatozoa with G-75FR is shown in Fig. 3. Maximum values for InsP_3 were obtained at 10 s with very rapid removal of this phosphate occurring over the following 20 s (Fig. 3*a*). The peak of InsP_2 production (Fig. 3*b*) was somewhat higher than that seen for InsP_3 and was reached a little more slowly (after about 30 s). A noticeable increase in Ins$P$ (Fig. 3*c*) was not seen until 60 s, which coincided with a corresponding decrease in InsP_2. More highly phosphorylated forms of inositol (e.g. InsP_4) were not detected over the time frame used in these experiments. Addition of 250 μM-La^{3+} to sperm just prior to G-75FR virtually eliminated the release of inositol phosphates (Figs. 3*a*–3*c*).

Progesterone-induced increase in intracellular Ca^{2+}

Other work from this laboratory strongly suggested that at least 60% of the acrosome reaction-inducing ability of follicular fluid was due to progesterone, and that the dose of progesterone sufficient to induce the largest number of acrosome reactions was 1 μg/ml [9]. Later analysis of the G-75FR used for the experiments shown in Fig. 1 revealed a progesterone concentration of 3.4 μg/ml before concentration of the fraction [30]. If we assume that no losses occur during ultrafiltration, then the concentration of progesterone in this concentrated fraction would be 13.6 μg/ml (i.e. sperm were stimulated with 2.2 μg/ml in experiments shown in Fig. 1). We assessed the Ca^{2+}-mobilizing activity of progesterone by direct comparison with amounts of this G-75FR which we estimated would contain equivalent concentrations of progesterone (Fig. 4*a*). It can be seen from these traces

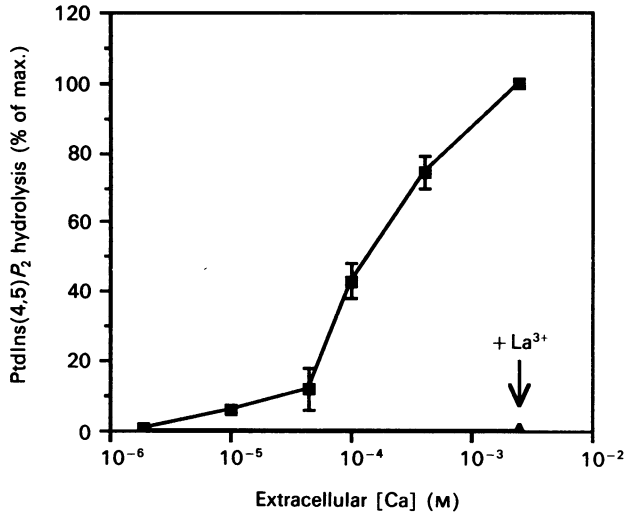

Fig. 5. Ca^{2+}-dependence of the progesterone-stimulated breakdown of PtdIns(4,5)P_2

Radiolabelled human sperm ($\sim 3.5 \times 10^7$/ml) were stimulated with 1 μg of progesterone/ml in the absence or presence of various chelator solutions (see the Materials and methods section) for 15 s at 37 °C and then extracted with methanol/chloroform/conc. HCl. PtdIns(4,5)P_2 was separated using t.l.c., and the relevant spots were scraped and the radioactivity counted. Each point is the percentage (average ± range) of the 'maximum breakdown' occurring in two separate experiments, maximum breakdown being that which occurred on stimulation of sperm with 1 μg of progesterone/ml in the absence of chelators (i.e. in the presence of 2.5 mM-Ca^{2+}). In each experiment controls (dimethyl sulphoxide/BSA vehicle) and sperm treated with progesterone in the absence of chelators were run in duplicate. Maximum breakdown in the two experiments was 30% and 37%; control sperm containing 920 d.p.m. of PtdIns(4,5)P_2/sample and 1145 d.p.m. of PtdIns-(4,5)P_2/sample in each experiment respectively. ■, Samples of sperm treated with 1 μg of progesterone/ml; ▲, sample treated with 1 μg of progesterone/ml in the absence of chelators but in the presence of 250 μM-La^{3+}. Motility after the labelling period in these experiments was 85%.

that progesterone can indeed mobilize Ca^{2+} and that maximal responses are obtained with a concentration of ~ 0.25 μg/ml (~ 0.8 μM). It is also apparent that the effects of progesterone and the G-75FR containing equivalent amounts of progesterone are almost identical. Studies by Siiteri *et al.* [7] were unable to detect acrosome reaction-inducing activity in organic extracts of G-75FR; however, the fractions obtained by Siiteri and coworkers were prepared by a slightly different procedure and probably did not contain as much progesterone as the fractions we have used for this and previous studies [8,30]. The inability of organic extracts of the G-75FR prepared by Siitteri *et al.* [7] to induce the acrosome reaction may have been due to those lower concentrations and/or to insufficient recovery of progesterone in their extraction/resolubilization procedures (see [30] for discussion). The results shown in Fig. 4 strongly suggest that progesterone is the major source of Ca^{2+}-mobilizing activity in the G-75FR used here and in the earlier work of Thomas & Meizel [8].

Inhibition of the progesterone-induced increase in intrasperm [Ca^{2+}] by La^{3+}

The increase in intrasperm Ca^{2+} stimulated by the G-75FR has previously been shown to be completely eliminated by 250 μM-La^{3+} [8]. Fig. 4(*b*) demonstrates that this concentration of La^{3+} also completely blocks the progesterone-induced increase in intrasperm Ca^{2+}, the Ca^{2+} response being half-maximal at about 50 μM-La^{3+} in the presence of 2.5 mM extracellular Ca^{2+}. This provides further support for the idea that progesterone is the major Ca^{2+}-mobilizing activity of G-75FR, and that progesterone acts by inducing an influx of Ca^{2+} from the extracellular space.

The dependence on extracellular Ca^{2+} of progesterone-induced PtdIns(4,5)P_2 hydrolysis

In the present study, when sperm were stimulated with 1 μg of progesterone/ml in the presence of varying concentrations of extracellular Ca^{2+}, PtdIns(4,5)P_2 hydrolysis was seen to be clearly dependent upon the [Ca^{2+}] (Fig. 5). Half-maximal breakdown of PtdIns(4,5)P_2 occurred at an extracellular [Ca^{2+}] of approx. 100 μM and was completely inhibited at 2 μM extracellular Ca^{2+}. Likewise, the addition of 250 μM-La^{3+} (in the presence of 2.5 mM-Ca^{2+}) completely blocked the breakdown of PtdIns(4,5)P_2 (Fig. 5). The breakdown of PtdIns4*P* stimulated by progesterone showed a similar dependence on extracellular [Ca^{2+}] and likewise was completely inhibited by 250 μM-La^{3+} (results not shown).

DISCUSSION

The results presented here demonstrate for the first time that phosphoinositide hydrolysis occurs in human sperm when stimulated by a potentially physiological stimulus, follicular fluid (G-75FR) or progesterone. The breakdown of these minor membrane phospholipids in human sperm in response to G-75FR/progesterone seems to be completely dependent upon the presence of extracellular Ca^{2+}. This finding is similar to results obtained in excitable tissues stimulated with acetylcholine [31] and hepatocytes stimulated with vasopressin [32]. Likewise, Domino & Garbers [18] demonstrated that production of inositol phosphates in sea-urchin sperm in response to the acrosome reaction inducer FSG was also dependent upon Ca^{2+} influx. These results do not support a role for phosphoinositide metabolism in the control of plasma-membrane Ca^{2+} influx in sperm. In sea-urchin sperm this is perhaps not too surprising as FSG-stimulated Ca^{2+} influx is thought to occur via a voltage-operated Ca^{2+} channel (VOCC) [11,33]. In mammalian sperm, however, K^+-induced depolarization does not stimulate acrosome reactions [34]. Similarly, the effects of VOCC antagonists on mammalian sperm acrosome reactions are somewhat contradictory [35–38]. In this respect we have observed no effect of verapamil (a phenylalkylamine-type VOCC blocker) on the follicular fluid-induced acrosome reaction (acrosome reactions: control, 8%, follicular fluid, 42%, follicular fluid + 100 μM-verapamil, 38%; $n = 2$), and likewise we have been unable to induce the acrosome reaction in human sperm by K^+-induced depolarization (P. Thomas & S. Meizel, unpublished work).

Progesterone is generally thought to pass through the plasma membrane and act at the level of the nucleus [39].

Nevertheless, it is clear from our previous work [8,9,30] and the results presented here that progesterone elicits a very rapid response in human sperm which (due to its speed and the lack of a cytoplasmic protein synthetic apparatus) must be independent of macromolecule synthesis. Steroids have previously been suggested to evoke non-genomic cellular responses via interactions at the cell surface [40], and progesterone has even been suggested to induce an increase in intracellular Ca^{2+} in *Xenopus* oocytes [41,42]; however, this finding is a matter of some controversy [43]. Our findings that progesterone can cause an influx of extracellular Ca^{2+} in sperm and induce a rapid exocytotic event [9] may provide some of the strongest evidence yet that steroids may act at the level of the cell surface. Additionally, our results may suggest a novel unifying mechanism (Ca^{2+} influx) by which progesterone can affect cellular functions other than through the transcriptional apparatus. Elucidation of the mechanism by which progesterone might elicit such a Ca^{2+} influx is therefore of the utmost importance.

Apart from VOCCs, the regulation of Ca^{2+} influx through the plasma membrane is poorly understood. It seems clear that a variety of ligands can control Ca^{2+} influx by means other than VOCCs [44,45]. Such ligand-gated channels have been placed into two separate categories: second-messenger-operated Ca^{2+} channels (SMOCCs), in which receptor and ion channel are separate entities coupled by a diffusible second messenger, and receptor-operated Ca^{2+} channels (ROCCs) in which the receptor and ion channel coexist as an individual functional unit [44].

Several SMOCCs have been identified; these include Ins(1,4,5)P_3-operated Ca^{2+} channels of lymphocytes [15] and mast cells [16] and a Ca^{2+}-operated Ca^{2+} channel in neutrophils [46]. Indirect evidence of a Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-regulated Ca^{2+} channel has also been presented [13,14]; however, this idea is still somewhat controversial [47,48]. The evidence presented here would suggest that neither a Ins(1,4,5)P_3 [or Ins(1,4,5)P_3/Ins(1,3,4,5)P_4]-operated channel nor a Ca^{2+}-operated channel are involved in the progesterone-stimulated Ca^{2+} influx in human sperm. It seems clear that Ins(1,4,5)P_3 generation is dependent upon Ca^{2+} influx, and we have no evidence that release of Ca^{2+} from an intracellular store occurs prior to Ca^{2+} influx in sperm stimulated with either progesterone or the G-75FR ([8], and see Fig. 4). With regards to ROCCs, the existence of a progesterone-operated Ca^{2+} channel remains to be determined.

Another potential mechanism by which an influx of Ca^{2+} might be obtained is by an inhibition of the plasma membrane Ca^{2+}-ATPase. Progesterone has been shown to bind to and inhibit the Ca^{2+}-ATPase of synaptosomal plasma membranes [49], and recently inhibitors of Ca^{2+}-ATPases have been demonstrated to accelerate the onset of the acrosome reaction in guinea pig sperm [50]. Fewtrell & Gomperts [51] have suggested that in mast cells certain ligands might uncouple the ATPase from its Ca^{2+}-translocating activity thus allowing a passive flow of Ca^{2+} through the transport protein into the cytosol. Further experimentation will be required to determine whether progesterone can modulate Ca^{2+}-translocating ATPases in this way.

Although the evidence presented here strongly suggests that progesterone acts on human sperm to initiate an influx of extracellular Ca^{2+} which in turn activates a polyphosphoinositide-specific phosphodiesterase, there is another scenario which could explain these results. It is possible that the interaction of progesterone with its putative sperm-surface receptor is dependent on Ca^{2+} and inhibited by La^{3+}. If this were the case then the data presented here could equally well support the hypothesis that progesterone binds to a cell-surface receptor which in turn activates phosphoinositide breakdown leading to Ins(1,4,5)P_3-mediated Ca^{2+} mobilization. Differentiation between these two possibilities will require further experimentation, but in defence of the hypothesis that progesterone stimulates Ca^{2+} influx in human sperm which then initiates phosphoinositide hydrolysis, it should be pointed out that binding of progesterone to the *Xenopus* oocyte plasma membrane receptor does not seem to be Ca^{2+}-dependent [42].

If we assume that follicular fluid-induced PPI hydrolysis in human sperm is Ca^{2+}-dependent, then the question arises as to what is the function of this phosphoinositide breakdown? Recent work of Roldan & Harrison [22] has suggested that PPI breakdown might play some role in the process of the acrosome reaction. A possible mode of action for such phospholipid hydrolysis would be the generation of diacylglycerol, which could then activate protein kinase C [52]. Protein kinase C has been suggested to play a role in the exocytotic process [53]; however, the presence of protein kinase C in some mammalian sperm has recently been questioned [54]. Likewise, the same study demonstrated no effect of phorbol esters, potent activators of protein kinase C, on the ionophore-induced acrosome reaction [54]. Additionally, treatment of human sperm with phorbol esters under a variety of conditions has no discernible effect on the acrosome reaction (P. Thomas & S. Meizel, unpublished work). Thus a potential role of PPI breakdown in activation of sperm protein kinase C is debatable.

The experiments with fura-2-loaded sperm reveal another interesting observation. It was originally found that G-75FR could stimulate Ca^{2+} influx in capacitated human sperm [8]. This influx of Ca^{2+} was shown to be required for stimulation of the acrosome reaction by the G-75FR [8]. Progesterone can also stimulate a rapid transient increase in intracellular [Ca^{2+}] in capacitated sperm, though the involvement of influx was not investigated [30]. However, it is clear from Fig. 4 that both G-75FR and progesterone can elicit Ca^{2+} influx in sperm that have not been capacitated. By definition, acrosome reactions do not occur in uncapacitated sperm (see [1]); correspondingly, neither follicular fluid [6] nor progesterone [30] stimulate acrosome reactions in uncapacitated human sperm. There are two alternative hypotheses which could explain why an inducer of the acrosome reaction (which evokes an increase in intracellular Ca^{2+}) fails to do so in uncapacitated spermatozoa: (1) the inducer fails to cause Ca^{2+} influx (perhaps because a receptor or channel is blocked), or (2) the inducer causes Ca^{2+} influx but the acrosome reaction does not occur because either a Ca^{2+}-responsive element is missing or some factor is blocking the exocytotic pathway downstream of Ca^{2+} mobilization. Our results therefore support the latter hypothesis. This suggests that changes occurring during capacitation, whatever they may be, somehow 'prime' the sperm to respond to the progesterone-induced Ca^{2+} influx.

In summary, follicular fluid/progesterone elicits a rapid Ca^{2+} influx in human sperm by a mechanism which seems to be independent of phosphoinositide hydrolysis.

As a result of the increase in intracellular [Ca^{2+}] there is a rapid breakdown of the PPIs which may play some, as yet unidentified, role in the process of the human sperm acrosome reaction.

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