Phospholipase C in mouse oocytes: characterization of β and γ isoforms and their possible involvement in sperm-induced Ca^{2+} spiking

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This study involved an investigation of the role of phospholipase C (PLC) in generating repetitive Ca^{2+} spikes at fertilization. Using a PCR-based strategy we have demonstrated that mouse oocytes have mRNA coding for PLCβ1, PLCβ3 and PLCγ isoenzymes. Furthermore, immunodetection of PLCγ1 using monoclonal antibodies reveals that PLCγ1 protein is present in mature mouse oocytes, ruling out the possibility that mRNA was being transcribed but not expressed. We were unsuccessful at detecting the presence of PLCβ protein, but the presence of this isoform can be inferred from functional studies. The PLC inhibitor, U73122, exerted an inhibitory effect on oocytes activated by spermatozoa or acetylcholine at concentrations of 10 and 30 μM respectively, while its inactive analogue had no effect. The soluble tyrosine kinase inhibitors, genistein (100 μM), herbimycin (10 μM) and geldanamycin (0.6 μM) which could affect signalling through PLCγ hindered but never completely inhibited Ca^{2+} spiking in response to fertilization. We conclude that the activation of PLC to generate $InsP_3$ may play a critical role in fertilization.

INTRODUCTION

In most mammalian oocytes, fertilization is followed by a series of cytosolic Ca^{2+} transients necessary to trigger oocyte development [1–7]. The first few Ca^{2+} transients occur as waves starting at the point of sperm attachment from where they travel across the whole oocyte [3,7]. The repetitive Ca^{2+} transients are due to a periodic release of Ca^{2+} from inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive stores since they can be initiated in the absence of external Ca^{2+} [8] and inhibition of the $InsP_3$ receptor by a monoclonal antibody which binds close to the Ca^{2+} release site inhibits the Ca^{2+} transients [9,10]. The transients appear to be associated with an increased influx of Ca^{2+}, which is essential for sustained spiking [8,11,12].

The transducing mechanism by which a sperm–oocyte interaction at the plasma membrane transfers a message to the intracellular Ca^{2+} stores remains to be elucidated [13]. Three main hypotheses have been proposed to explain how the spermatozoon induces Ca^{2+} release. The 'Ca^{2+} bomb hypothesis' proposes that some Ca^{2+}, carried in the sperm cytoplasm, is deposited into the ooplasm. There it activates the release of Ca^{2+} by sensitizing the $InsP_3$-sensitive Ca^{2+} channel and so initiates the first Ca^{2+} wave [14,15]. However, the intracellular microinjection of Ca^{2+} does not reproduce the normal repetitive Ca^{2+} transients observed during natural fertilization, making this hypothesis unlikely [8,16].

A second hypothesis suggests that the spermatozoon might introduce, after its fusion with the oocyte, a soluble factor which would induce Ca^{2+} release from the intracellular stores [17–19]. A protein factor extracted from sperm and injected into the oocyte is able to induce Ca^{2+} oscillations resembling those observed at fertilization [20].

Finally, it has been proposed that the spermatozoon may bind to a receptor and activate a G-protein-coupled phospholipase C (PLC), thereby generating $InsP_3$ which triggers the release of Ca^{2+} from intracellular stores [14,21,22]. Elements of this pathway certainly exist in hamster and mouse oocytes since repetitive Ca^{2+} transients can be induced by acetylcholine (ACh) and 5-hydroxytryptamine respectively, hormones known to activate PLCβ, the isoform coupled to G-proteins [7,23,24]. In addition, repetitive Ca^{2+} transients can be induced by the iontophoretic injection of either non-hydrolysable GTP analogues [22] or $InsP_3$ into mouse and hamster oocytes [9,25] and by electroporation of $InsP_3$ into mouse oocytes [26]. However, an involvement of the pathway in the fertilization response is usually cast aside because phorbol esters, while inhibiting Ca^{2+} oscillations induced by guanosine 5′-[γ-thio]triphosphate (GTP[S]), have little effect on those induced by fertilization [23,27]. An alternative route by which sperm attachment might increase the level of $InsP_3$ is to activate PLCγ.

In the present study, we have focused on this proposed involvement of PLC in Ca^{2+} spiking at fertilization. The mammalian PLC family can be divided into three types β, γ and δ [28]. Each type is subdivided into several isoenzymes: β1, β2, β3, γ1, γ2, δ1, δ2, δ3. The three isoforms differ greatly in their amino acid sequence but they all possess two regions of homology, the so-called X and Y boxes. Between the three PLC isoforms, these X and Y regions are respectively about 60 % and 40 % identical. They may constitute the catalytic domain of the phospholipase. In addition to the X and Y boxes, PLCγ contains the characteristic *src* homology domains SH2 and SH3 [29]. The X, Y, SH2 and SH3 regions of the PLC amino acid sequence contain several constant peptides throughout the PLC family and these were used as the basis for a PCR detection strategy. Using this strategy, mRNA encoding the PLCβ1, PLCβ3 and PLCγ isoforms was detected in mouse oocytes. A role for PLC has also been examined using the aminosteroid U73122 which efficiently inhibits PLC activity in platelets [30], neuronal cells [31],

Abbreviations used: ACh, acetylcholine; hCG, human chorionic gonadotrophin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $InsP_3$, inositol 1,4,5-trisphosphate; DTT, dithiothreitol; GDP[S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; PLC, phospholipase C; PVP, polyvinylpyrrolidone.

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neuroblastoma × glioma hybrid cells [32], and kidney cells [33]. We conclude that the activation of PLC to generate InsP_3 may play a critical role in fertilization.

MATERIALS AND METHODS

PCR protocols

A nested primer PCR strategy was used to amplify PLCβ and PLCγ cDNAs. For PLCβ, degenerate sense and antisense oligonucleotides corresponding to amino acids QQAKMEAY and WN(A/N)CTCQL respectively of the conserved X and Y regions of PLCβ were synthesized (Pharmacia; Table 1). For the nested PCR, the sense oligonucleotide used in the first run was conserved and a new degenerate antisense primer corresponding to amino acids MPQLFWN was synthesized (Table 1). An *Eco*R1 and a *Sal*1 restriction site were included in the sequence of the nested sense and antisense primers respectively to facilitate later subcloning (Table 1). For PLCγ, degenerate primers based on the amino acid sequences of the SH3 and Y regions were synthesized (Pharmacia). These corresponded to amino acids WWRGDYG (sense) and MQMNQAL (antisense) respectively (Table 1). The nested primers coded for the peptides FRSNYV (sense) and LNFQTP (antisense). An *Eco*R1 and a *Bam*H1 restriction site were included in the sequence of the nested sense and antisense primers respectively to facilitate later subcloning (Table 1).

Messenger RNA was prepared from mouse oocytes or spermatozoa using a kit purchased from Pharmacia (QuickPrep Micro mRNA purification kit) following the manufacturer's instructions. Reverse-transcriptase PCR (RT-PCR) was performed on mRNA from 150 and 370 mouse oocytes for PLC-β and -γ respectively or from spermatozoa expelled from the epididymides. Reverse transcription was carried out in 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 unit spermidine, 10 mM dithiothreitol (DTT), 1 mM dNTPs, 20 units RNase inhibitor, 0.5 μg of hexaprimers and 0.5 μl of AMV reverse transcriptase (Promega). After incubation at 37 °C for 1 h, the sample was heated for 10 min at 94 °C to denature the reverse transcriptase. Control reactions contained the same amount of mRNA as experimental reactions but no AMV reverse transcriptase was added. PCR reagents were then added to the same tube to give a final concentration of 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs and each primer at 1 μM in a volume of 100 μl. The PCR samples were heated to 94 °C (3 min) and *Taq* polymerase (2.5 units; Boehringer) was added. Amplification was carried out in a Biometra UNO-thermoblock for 30 cycles as follows: denaturation at 94 °C for 1 min; primer annealing at 55 °C for 2 min; and chain extension at 72 °C for 1 min 30 s. A one-hundreth volume of this reaction was reamplified using the nested primers (1 μM) under the same conditions as above except that the cycling parameters were: denaturation at 94 °C for 1 min; primer annealing at 55 °C for 40 s; and chain extension at 72 °C for 1 min.

Subcloning and sequencing

PCR products were fractionated by agarose gel electrophoresis and purified by β agarase I gel digestion. Purified DNA was digested by either *Eco*R1/*Sal*1 (PLCβ) or *Eco*R1/*Bam*H1 (PLCγ) endonucleases and subcloned into the corresponding sites of pBluescript KS (Stratagene). Sequencing was performed on double-stranded plasmid DNA using Sequenase Version 2 (Amersham) according to the manufacturer's instructions. Four to five individual clones were sequenced for each PCR product.

SDS/PAGE and Western blotting analysis of proteins

Whole oocytes or spermatozoa were solubilized by boiling (3 min) in Laemmli's [34] gel sample buffer. Proteins were separated by electrophoresis on an SDS/6 %-polyacrylamide gel under reducing conditions. Proteins were then transferred electrophoretically on to a nitrocellulose membrane using a semi-dry blotter apparatus (Biometra). The membrane was probed with a mixture of six different monoclonal anti-PLCγ1 antibodies (mAbγ1; [35]). Immunodetection was carried out using the enhanced chemiluminescence reaction (ECL) kit (Amersham), according to the manufacturer's instructions.

Oocytes and spermatozoa

MF1 female mice (3–4 weeks; OLAC, Bicester, U.K.) were superovulated by intraperitoneal injection of 5 or 10 i.u. of pregnant mare's serum gonadotrophin (PMS; Intervet, Cambridge, U.K.) followed 48 h later by 5 or 10 i.u. of human chorionic gonadotrophin (hCG; Intervet). Unfertilized oocytes (12–13 h post-hCG) were released from the oviduct into warmed medium H6 containing 4 mg/ml BSA (a Hepes-buffered form of modified T6 medium [36]). Cumulus cells were removed by brief exposure to hyaluronidase (0.1 mM; Sigma) and zonae pellucidae removed by exposure to α-chymotrypsin (0.001 %; Sigma type II; [37]). Oocytes were held in drops of H6 + BSA under paraffin oil (FSA Laboratories, Loughborough, U.K.) in Falcon tissue-culture dishes. All manipulations were carried out at 37 °C on heated stages, pads or in incubators.

Spermatozoa were expelled from the vas efferentia and cauda epididymides of male CFLP mice into 1 ml of Whittingham's medium [38] containing 30 mg/ml BSA and incubated under oil for 1–3 h at 37 °C and 5 % CO_2 to capacitate. Aliquots of 40–100 μl were taken for insemination of oocytes.

Oocytes were fertilized either by insemination of zona-free oocytes attached to the base of chambers on the warmed

Table 1 PCR primers

Set of primers selected for the amplification of a region in PLCβ (1st and 2nd rows) or PLCγ (3rd and 4th rows). The slashes in some of the primer sequences (wobbles) indicate the use of a mixture of forward and/or reverse primers in order to obtain a complete match with each template. N = A/C/T/G, I = Inosine. The co-ordinates for localization refer to the rat (r) PLCβ [70] and human(h) PLCγ1 [71] sequences. The reverse primers are indicated as the reverse complement.

Type		Forward primers		Reverse primers	
		Sequence	Localisation	Sequence	Localisation
PLCβ	1st run	CA(G/A)CA(G/A)GCIAA(G/A)ATGGCNGA(A/G)TA	r1258-1280	CAT(C/T)TG(A/G)CAICCNGC(G/A)TTCCA	r1843-1863
	nested	ATATGAATTCCA(G/A)GCIAA(G/A)ATGGCNGA(A/G)TA	r1258-1280	ATATGTCGACTTCCA(A/G)AANA(G/T/A)(C/T)TGIGGCAT	r1828-1848
PLCγ	1st run	TGGTGG(C/A)GNGGIGA(C/T)TA(T/C)GG	h2481-2500	ATATGAATTCTGGTGG(C/A)GNGGIGA(C/T)TA(T/C)GG	h3141-3159
	nested	ATATGAATTCCCCTCAAACTATGTGG	h2523-2451	ATATGGATCCTGGGGTCTGGAAATTGAG	h3114-3129

microscope stage (see below), or, when a longer interval between fertilization and analysis of Ca^{2+} spiking was desired, in drops of T6+BSA medium under oil. These latter oocytes were then transferred to chambers.

Intracellular bivalent cation measurements

Eight to 20 zona-free oocytes were washed and transferred to H6 medium + polyvinylpyrrolidone (PVP; 6 mg/ml) on a coverslip which had been precoated with concanavalin A (Con A; 0.2 mg/ml in PBS) and which formed the base of a metallic perfusion chamber [39]. Oocytes were then loaded with fura-2 acetoxymethyl ester (2 μM; Molecular Probes) for 20 to 30 min and washed extensively with H6+PVP. The chamber was then placed in a well on the stage of a Nikon Diaphot TMD inverted epifluorescence microscope for imaging. Solutions were introduced via a system of continuous perfusion through the chamber maintained at 37 °C.

Intracellular free bivalent cation activity was imaged through a Nikon CF-Fluor ×20 objective and intensified CCD camera (Extended ISIS, Photonic Science, Robertsbridge, U.K.), by calculating the ratio of fura-2 fluorescence at 510 nm, excited by UV light alternately at 340 and 380 nm from twin Xenon arc lamps and grating monochromators. Excitation wavelengths were alternated by a rotating chopper mirror attached to a stepper-motor, which was driven in synchrony with the video signal from the camera, to switch wavelengths at the end of each video frame. The resulting video signals were combined by an 'Imagine' digital image processor (Synoptics Ltd., Cambridge, U.K.) using a lookup table to implement the formula of Grynkiewicz et al. [40]. The calculation was done in real time, to give a 'live' image of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which was updated every 80 ms, and smoothed by recursive filtering with a 200 ms time-constant to reduce the noise [39,41].

In all cases the live image was recorded continuously on video tape, and played back subsequently and redigitized into a framestore, using software written in the semper language (Synoptics Ltd.) to sample selected oocytes and to record and plot either mean $[Ca^{2+}]_i$ or fluorescence readings at regular time intervals. In most cases, data were sampled at 4 or 5 s intervals, but for more detailed analysis sampling could be speeded up to 0.8 s.

To determine whether fura-2-loaded oocytes had been fertilized, the coverslip was examined in the chamber under bright field for evidence of polar body extrusion.

Materials

ACh, thimerosal and $CaCl_2$ were all from Sigma. Fura-2 acetoxymethyl ester was from Molecular Probes. U73122 and U73343 from Cascade, were dissolved in chloroform (0.3% final concentration). The mAbγ1 and mAbγ2 mixtures were a kind gift from Dr. Soo Goo Rhee.

RESULTS

PCR characterization of PLCβ1 and PLCβ3 in mouse oocytes

PCR amplification of mouse oocyte cDNA was performed using primers corresponding to DNA sequences localized within the X and Y boxes of the enzyme, but not specific for the β1, β2 and β3 isoforms (Table 1). The PCR reaction could therefore amplify any isoform of the enzyme. Agarose gel electrophoresis of the products of the first round of amplification revealed a fragment of 700 bp (Figure 1A; lane b). Reamplification of this cDNA using the nested primers (Table 1) led to a clearly visible signal in the form of two bands of 700 and 600 bp (Figure 1, lane c). The smaller fragment corresponded to the predicted size of the β1 isoform. The larger, 700 bp fragment was predicted to be the β3 isoform as the region between the X and Y boxes is greater than for PLCβ1 [42]. DNA sequencing confirmed the identity of these two fragments. Figure 1(B) (box 1) shows an alignment of the predicted amino acid sequence encoded by the 680 bp fragment with that of rat brain PLCβ1. In the region limited by the primers the mouse oocyte β1 fragment shared 97% amino acid identity and 94% DNA similarity with the rat brain sequence. A comparison of the predicted amino acid sequence for β3 with rat brain PLCβ3 is shown in Figure 1(B) (box 2). The two sequences show 95% amino acid identity and 92% DNA identity in the region limited by the PCR primers. An alignment of the mouse oocyte β1 and β3 sequences is shown in Figure 1(B) (box 3). One can see between the X and Y boxes the additional peptide characteristic of the β3 isoform. The mouse oocyte β1 fragment shows 51% identity with the mouse oocyte β3 fragment. Attempts to detect PLCβ proteins by immunoblotting were not successful despite the detection of PLCβ in control cells.

Presence of PLCγ1 RNA message and the fully translated protein in mouse oocytes

PCR amplification of mouse oocyte cDNA was carried out using primers complementary to DNA sequences localized within the SH3 and Y boxes of PLCγ (Table 1). Agarose gel electrophoresis of the reaction revealed a fragment of the predicted size ($\approx$ 640 bp; Figure 2A, lane b). A subsequent round of amplification using nested primers did not reveal any further products (Figure 2, lane c). DNA sequencing revealed that this product codes for the γ1 isoform of PLC (Figure 2B). The sequence shows 98% identity with human brain PLCγ1 at the amino acid level and 89% similarity at the DNA level.

The presence of fully translated PLCγ1 protein was investigated by probing a Western blot of mouse oocytes with a mixture of monoclonal anti-γ1 antibodies (mAbγ1). A single band of molecular mass 150 kDa corresponding to PLCγ1 was detected (Figure 3, lane b). PLCγ2 could not be detected in mouse oocytes using an antibody to this isoform (mAbγ2) which is consistent with the observation that PCR resulted in only one amplification product. PLCγ1 was also detected in mouse spermatozoa expelled from the epididymides (Figure 3, lane a). However, as the sperm were not purified, this band could also contain PLCγ present in contaminating leucocytes and other cells.

Attempts were made to examine whether or not PLCγ1 was activated following fertilization. It is known that, on activation, PLCγ1 becomes phosphorylated on tyrosines 771, 783 and 1254 [43–45]. Therefore the phosphorylation state of the enzyme was compared before and after fertilization. Four hundred oocytes (4000 ng of protein) were labelled with ^{32}P and immunoprecipitation of PLCγ1 was carried out 18 min after insemination (average latency of the Ca^{2+} spiking response). It was not possible to detect any phosphorylation of PLCγ1 linked to the fertilization of oocytes.

Effects of U73122 on ACh-induced changes in cytosolic free Ca^{2+} in mouse oocytes

As it is well established that ACh stimulates Ca^{2+} spiking through PLC activation [7,24], we determined whether the PLC inhibitor U73122 could inhibit ACh-induced Ca^{2+} spiking in mouse eggs. As illustrated in Figure 4(A), oocytes respond to stimulation by 50 μM ACh with a series of transient rises in cytosolic free Ca^{2+}. There was an average of 3.3 spikes; the mean amplitude of the first one is equal to 150 nM ($n = 16$). A 30 min preincubation of

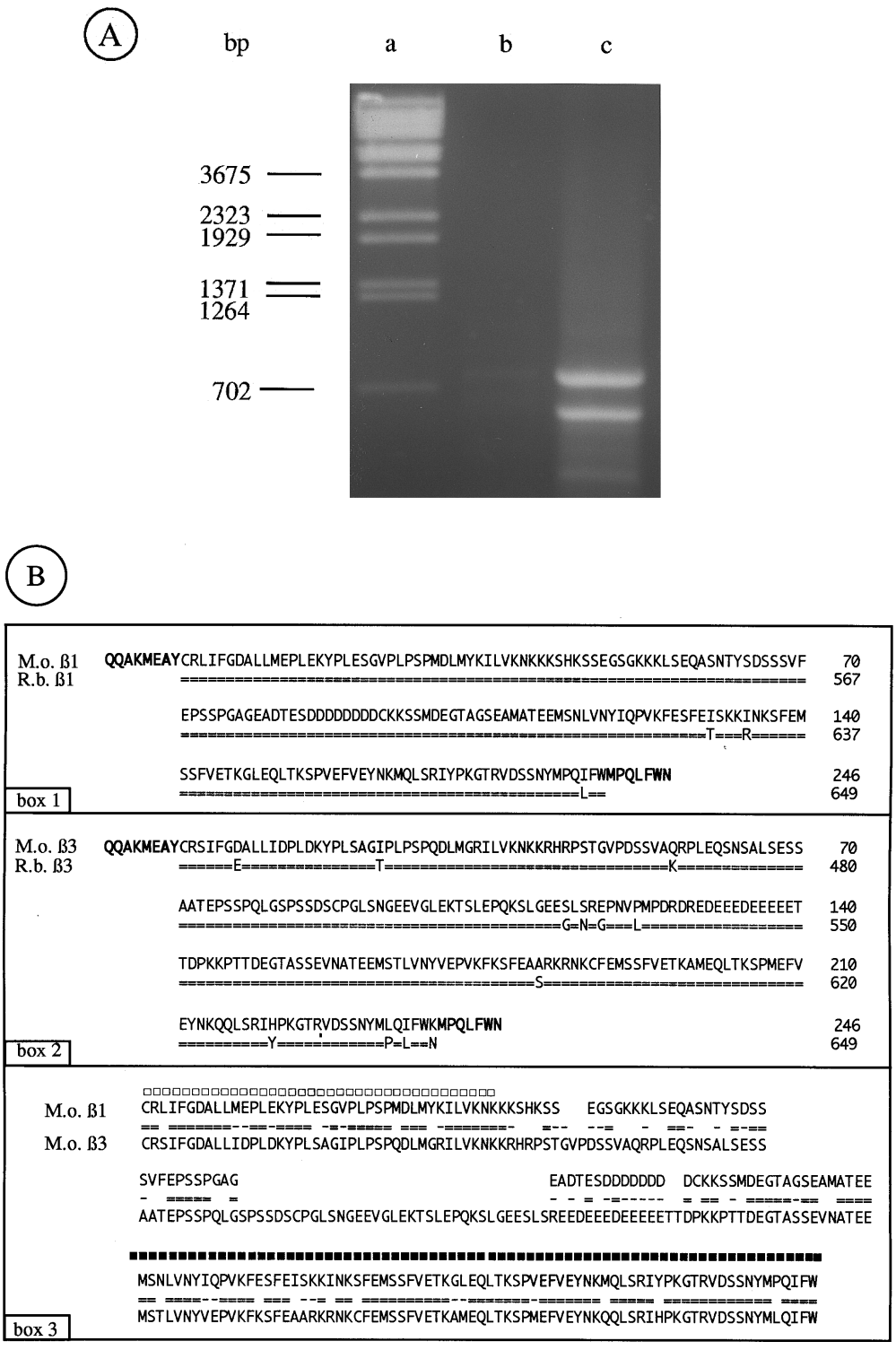

Figure 1 Detection of PLCβ1 and PLCβ3 mRNAs by RT/PCR

(**A**) mRNA prepared from 150 mouse oocytes, corresponding to 55 ng of total RNA [72], was subjected to RT/PCR using PLCβ-specific primers as described in the Materials and methods section. The RT/PCR was analysed by agarose gel electrophoresis on a 1% gel followed by ethidium bromide staining. Lanes: a, molecular-mass markers; b, mouse oocyte cDNA; c, reamplification of products from lane b using nested primers. (**B**) Amino acid sequence alignment of mouse oocyte (M.o.) PLCβ1 and rat brain (R.b.) PLCβ1 ([70]; box 1) and mouse oocyte PLCβ3 and rat brain PLCβ3 ([42]; box 2). Box 3 shows the amino acid sequence alignment of mouse oocyte PLCβ1 and mouse oocyte PLCβ3. Identical amino acids are marked by equal signs, and conservative substitutions are indicated by dashes. The internal extremities of the X and Y domain of PLCβ are indicated by the open squares and closed squares respectively. PCR primers are in bold type. Sequences have been deposited in the GenBank data base (*M. musculus* mRNA for PLCβ1 has accession no. X95344 and *M. musculus* mRNA for PLCβ3 has accession no. X95345).

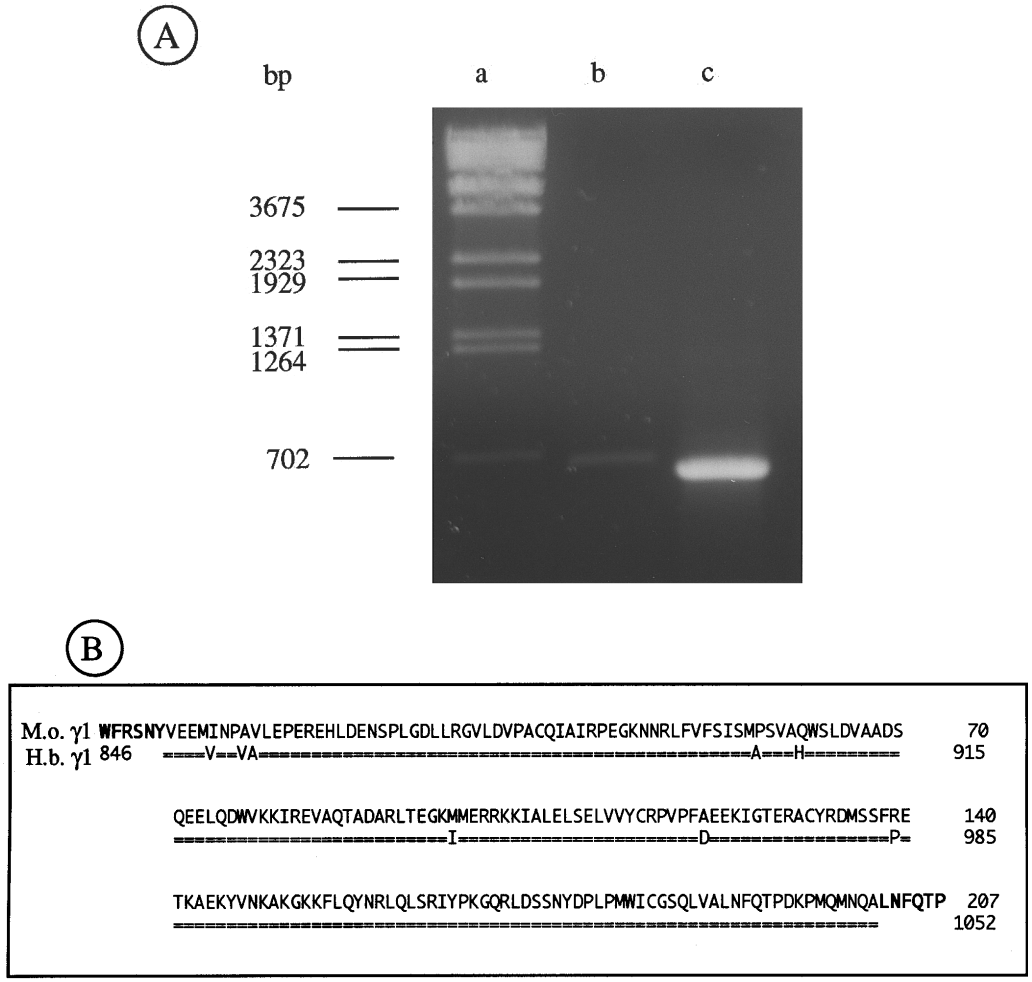

Figure 2 Detection of PLCγ1 by RT/PCR

(**A**) mRNA prepared from 370 mouse oocytes (130 ng of total RNA) was subjected to RT/PCR using PLCγ-specific primers as described in the Materials and methods section. The RT/PCR reaction was analysed by agarose gel electrophoresis on a 1% gel followed by ethidium bromide staining. Lanes: a, molecular-mass markers; b, mouse oocyte cDNA; and c, reamplification of products from lane b using nested primers. (**B**) Amino acid sequence alignment of mouse oocyte (M.o.) PLCγ1 and human brain (H.b.) PLCγ1 [71]. Identical amino acids are marked by equal signs. PCR primers are in bold type. The *M. musculus* mRNA sequence for PLCγ1 has been deposited in the GenBank database (accession no. X95346).

the eggs with the PLC inhibitor (30 μM) greatly reduced their Ca^{2+} responsiveness to ACh ($n = 14$; Figure 4B). The average number of spikes was reduced to 1.1 and the first spike amplitude dropped to 60 nM, which is very close to the basal level. Finally, when the concentration of U73122 was reduced to 10 μM ($n = 61$), both the mean amplitude of the first spike (116 nM) and the mean number of Ca^{2+} spikes (1.6) were slightly reduced compared with the control stimulation in the absence of inhibitor. It can thus be concluded that U73122 inhibits the response of mouse oocytes to ACh, most probably by inhibiting PLC activity.

Non-specific effects of U73122 have been reported in the literature. It has been shown that in pancreatic acinar cells, U73122 inhibits ATP-dependent Ca^{2+} uptake into the InsP_3-sensitive Ca^{2+} store, so depleting the store and causing a transient rise in cytosolic Ca^{2+} [46]. This inhibition does not appear to occur in mouse oocytes, as their perfusion with U73122 alone does not affect the resting Ca^{2+} level (results not shown). It was possible that U73122 inhibited Ca^{2+} oscillations in mouse oocytes by affecting the InsP_3 receptor/Ca^{2+} channel [46]. To exclude this possibility, oocytes spiking in response to thimerosal were perfused with U73122. As thimerosal stimulates Ca^{2+} spiking by sensitizing the InsP_3 receptor to basal InsP_3 levels [47], sustained oscillations induced by thimerosal would certainly be affected by any treatment targeting this receptor. U73122 had no effect on repetitive Ca^{2+} spiking induced by thimerosal ($n = 13$; Figure 4C), thereby excluding any effect of U73122 on events downstream of InsP_3 formation.

Effects of U73122 and U73343 on sperm-induced changes in cytosolic free Ca^{2+} in mouse eggs

Contact of spermatozoa with mouse oocytes provokes repetitive Ca^{2+}-spiking in the oocytes (Figure 5A). The initiation of Ca^{2+} spiking begins on average around 18 min after insemination and spiking can last for 2–4 h. As observed for ACh, the first sperm-induced spike is broader and has a larger amplitude than subsequent spikes (Figure 5A). Preincubation of mouse oocytes with U73122 (1 to 20 μM) for 30 min before sperm insemination caused a dose-dependent inhibition of the Ca^{2+} spiking in

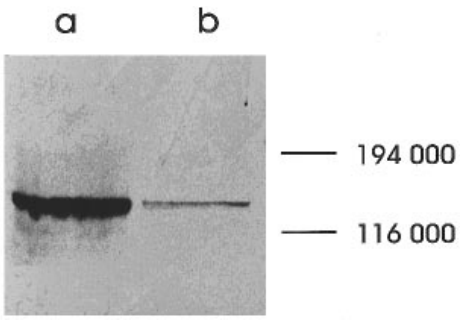

Figure 3 Immunodetection of PLCγ1 in mouse oocytes and spermatozoa

Either 150 mouse oocytes or 10 μl of non-diluted sperm (expelled from epididymides) were solubilized in Laemmli's buffer and proteins separated by SDS/PAGE electrophoresis (6% acrylamide). Proteins were blotted on to nitrocellulose membranes. Membranes were blocked for 1 h at 37 °C in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 5% fat-free dry milk. Transferred proteins were reacted with mAbγ1 (1/1000 dilution) in the same buffer [except, BSA (1%) was used instead of milk], overnight under mild agitation at 4 °C. Washes were 8 × 15 min in TBS/Tween/milk. Immunodetection was performed using the enhanced chemiluminescence technique (Amersham). The exposure time was 1 min. Lanes: a, mouse spermatozoa proteins; b, protein from 150 oocytes.

response to fertilization (Figure 6). An identical preincubation of oocytes with U73343, an inactive analogue of the PLC inhibitor U73122, had no effect on the Ca^{2+} response after fertilization. The IC_{50} for this inhibitory process was about 2.3 μM. Control experiments revealed that high doses of U73122 (10 μM) actually inhibited sperm fusion (by approx. 40%) and reduced the extent of polyspermy, while the inactive analogue had no effect on sperm fusion. In subsequent experiments, U73122 was therefore added after the repetitive spiking in response to fertilization had started. Two different types of response were observed. Using one batch of inhibitor, the Ca^{2+} spikes gradually declined in amplitude and then disappeared (Figure 5B). The complete disappearance of Ca^{2+} spiking occurred with a mean time of 45 min ($n = 15$). With a second batch of inhibitor, spiking stopped abruptly after the first few spikes in all oocytes (Figure 5C; $n = 9$). In the latter case, there was some hint of reversibility, because spiking recommenced in two oocytes after washout of the inhibitor (results not shown). In the absence of the inhibitor, spiking continued for 2 h or more. The difference in amplitude and frequency between Figures 5(A), 5(B) and 5(C) is in the usual range of variability between individual eggs [16].

Effect of tyrosine kinase inhibitors on sperm-induced changes in cytosolic free Ca^{2+} in mouse eggs

As PLCγ1 is activated by tyrosine phosphorylation, the effect of tyrosine kinase inhibitors on the ability of the sperm to induce Ca^{2+} spiking in mouse oocytes was tested. Three different compounds reported to inhibit the activity of *src*-like soluble tyrosine kinases were chosen: genistein, herbimycin and geldanamycin. Each inhibitor was used at the pre-established maximal dose [48–50]. Oocytes were preincubated for 30 min and inseminated in medium containing the inhibitor. Results of these experiments are reported in Table 2.

None of the three inhibitors was able to completely prevent Ca^{2+} spiking induced by fresh sperm, although they did have some clear effects. While all the oocytes in 100 μM genistein produced some spikes, a smaller percentage of the oocytes spiked in response to insemination in the presence of herbimycin and geldanamycin. Secondly, two of the three inhibitors (genistein

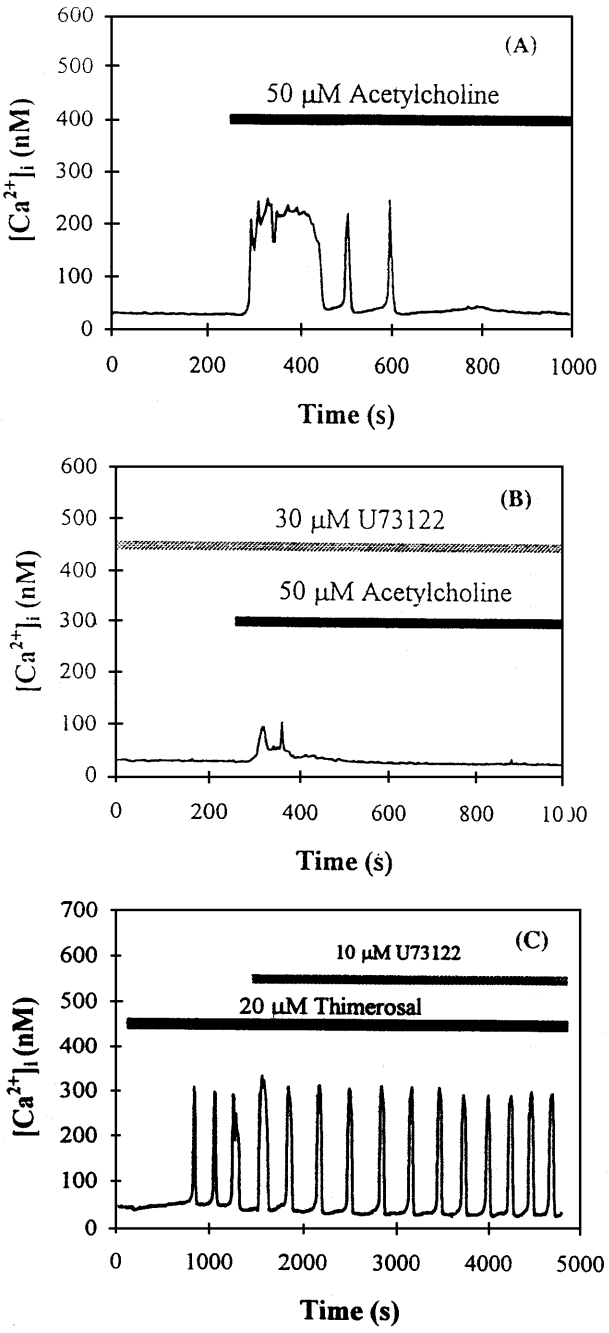

Figure 4 Effects of the PLC inhibitor U73122 on Ca^{2+} spiking induced in mouse oocytes by ACh and thimerosal

The graphs plotted are representative of typical responses. (**A**) Ca^{2+} spiking in response to ACh (50 μM; solid bar). (**B**) The effect of U73122 (hatched bar; 30 μM) on Ca^{2+} spiking in response to ACh (50 μM). In this case, oocytes were preincubated with the inhibitor 20 min before Ca^{2+} measurements were made. (**C**) The effect of U73122 (hatched bar; 10 μM) on Ca^{2+} spiking in response to thimerosal 20 μM (solid bar).

and geldanamycin) were able to abort the Ca^{2+} spiking process once it had started. Thus, when Ca^{2+} spiking was initiated in the presence of genistein or geldanamycin, $[Ca^{2+}]_i$ returned to and remained at its basal level after about three or four spikes. Finally, preincubation of the oocytes with all three inhibitors always led to an increase in the latency between sperm addition and the onset of the first spike. The inhibitors lengthened this

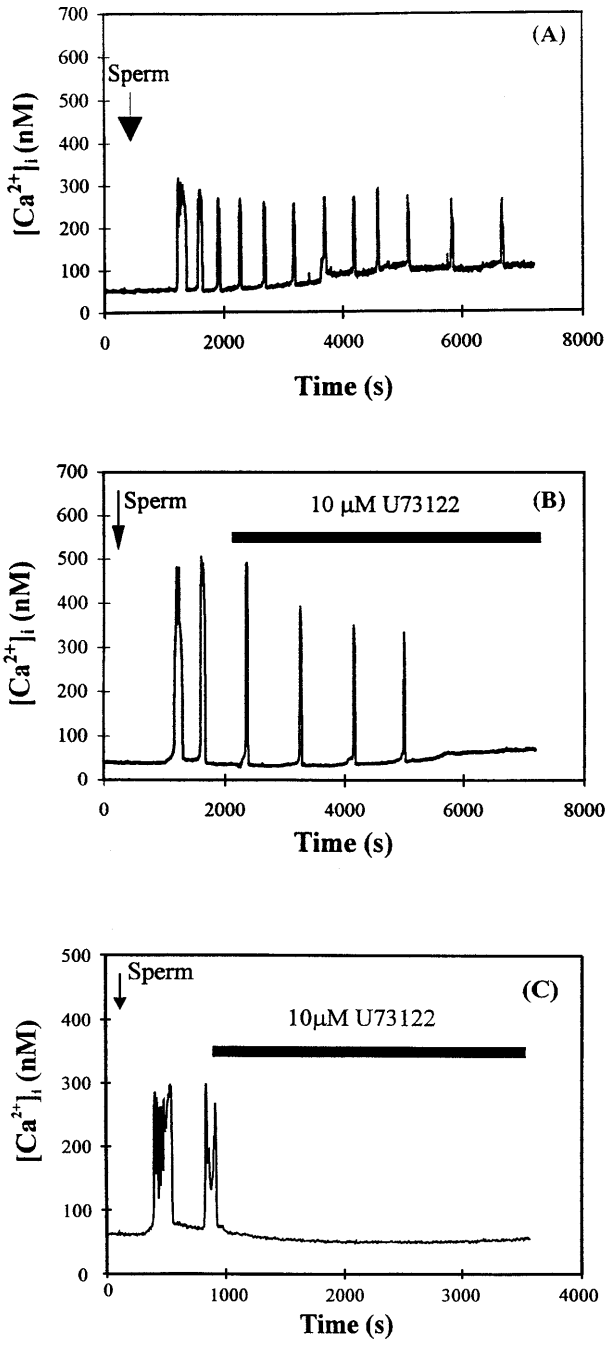

Figure 5 Effects of the PLC inhibitor U73122 on Ca^{2+} spiking at fertilization

Spermatozoa were added at arrow. (**A**) Fertilization Ca^{2+} train. (**B**) and (**C**) Effects of U73122 on the fertilization-induced Ca^{2+} spiking.

time lag from a mean of 18 min in the controls to about 30 min (Table 2). In summary, it appears that normal Ca^{2+} spiking in mouse oocytes is hindered by tyrosine kinase inhibitors, but never inhibited completely.

In a separate series of experiments, agonists such as insulin and fibroblast growth factor at concentrations known to give a physiological response in other cell types were not able to induce Ca^{2+} spiking when applied to mouse oocytes for extended periods (results not shown).

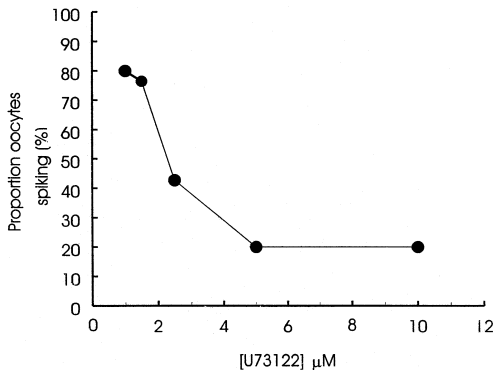

Figure 6 Effect of the PLC inhibitor U73122 on Ca^{2+} spiking at fertilization

Effect of preincubating oocytes for 30 min with various doses (1–10 μM) of the PLC inhibitor U73122 on Ca^{2+} spiking in response to fertilization. There was no inhibition of spiking following an identical preincubation of oocytes with U73343, the inactive analogue of this PLC inhibitor, at a concentration of 10 μM.

Table 2 The Ca^{2+} response at fertilization in mouse oocytes in the presence of three different inhibitors of soluble tyrosine kinases

In all cases, oocytes were preincubated 20–30 min before insemination in the concentrations of the inhibitors indicated. In the last column the average number of times the oocyte spiked in response to insemination is indicated in parentheses.

	Latency (min)	Percentage of oocytes spiking after insemination	Percentage of oocytes in which spiking was aborted
100 μM Genistein ($n = 15$)	28.6	100	73 (3.3 spikes)
10 μM Herbimycin ($n = 40$)	31.4	60	0
0.6 μM Geldanamycin ($n = 20$)	29.8	80	75 (4 spikes)

DISCUSSION

Using a PCR-based strategy we have demonstrated that mouse oocytes have mRNA coding for PLCβ1, PLCβ3 and PLCγ isoenzymes. Furthermore, immunodetection of PLCγ1 using monoclonal antibodies reveals that PLCγ1 protein is present in mature mouse oocytes (Figure 2), ruling out the possibility that mRNA was being transcribed but not expressed. We were unsuccessful at detecting the presence of PLCβ protein, but the presence of this isoform can be inferred from functional studies as described below. The demonstration that mouse oocytes contain PLC does not necessarily mean that the phosphoinositide signalling pathway is involved in fertilization. A role for $InsP_3$ has been supported by the finding that the mass of this second messenger increased from 53 to 261 fmol/egg following the fertilization of *Xenopus* oocytes [51]. As such direct measurements of $InsP_3$ levels in the very much smaller mammalian oocytes are technically impossible, the involvement of $InsP_3$ in mammalian fertilization has been addressed using indirect methods.

Previous studies using an antibody directed against the $InsP_3$ receptor have suggested that $InsP_3$ plays a direct role in fertilization in mouse [10] and in hamster oocytes [9]. We have supplied further evidence for such a role by studying the effects

of the PLC inhibitor U73122 and its inactive counterpart U73343. U73122 has already been shown to inhibit PLC activity in several tissues [30–33]. In this study, U73122 inhibited Ca^{2+} oscillations in mouse oocytes in response to ACh, the stimulatory effect of which is normally transduced through PLCβ via G$\alpha_{q/11}$ [52]. A non-specific action of the inhibitor on either Ca^{2+} pumps or the InsP_3 receptor was ruled out by showing that the inhibitor had no effect on thimerosal-induced calcium spiking. U73122 also exerted an inhibitory effect on oocytes activated by spermatozoa. When the inhibitor was added once Ca^{2+} oscillations had started two responses were observed depending on the batch of inhibitor used. The spikes either stopped abruptly upon addition of the inhibitor, or they declined slightly in amplitude and stopped after a mean time of 45 min; in the control situation Ca^{2+} oscillations continued for 1–2 h. Addition of U73122 prior to insemination prevented spiking, although part of this effect may have been due to a reduction in the sperm/oocyte fusion rate. Since our results suggest that the spermatozoon may contain PLCγ (Figure 3) it is also possible that this sperm enzyme may contribute to events at fertilization. Mammalian sperm has been shown to contain most of the elements of the phosphoinositide signalling cascade, e.g. G$\alpha_{q1/11}$, PLCβ and InsP_3 receptors [53]. At the time of fertilization, these signalling components will be added to the oocyte and could contribute to the generation of the InsP_3 which may be responsible for the onset of fertilization. Elements of this signalling cascade may represent the factor which may be isolated from sperm and can induce Ca^{2+} oscillations when injected into oocytes [20].

Overall, the results of the molecular and inhibitor experiments are consistent with the conclusion that after sperm fusion PLC is activated to promote Ca^{2+} release via InsP_3 formation and that oscillations are linked to the continuous synthesis of InsP_3 (Figure 5). Turning off the phosphoinositide signalling pathway, either by using antibodies directed against the InsP_3 receptor [9], or by using an inhibitor directed against PLC leads to inactivation of Ca^{2+} spiking.

The molecular studies have revealed that this putative role for PLC in fertilization could be mediated by either PLCβ (1 or 3) or PLCγ1. PLCβ isoforms 1, 2 and 3 are regulated by either Gα or G$\beta\gamma$ subunits [52,54]. *In vitro* analysis of the regulation of PLCβ isoforms by G-protein α and $\beta\gamma$ subunits has shown that G$\beta\gamma$ stimulates PLCβ3 most and PLCβ1 least, whereas G$\alpha_{q/11}$ subunits stimulate PLCβ1 most and PLCβ3 least [54,55]. Evidence implicating a G-protein in the initiation of the fertilization Ca^{2+} spikes is that injection of guanosine 5′-[β-thio]diphosphate (GDP[S]), a GTP-binding protein antagonist, inhibits this response in hamster oocytes in a dose-dependent manner [22]. Although they did not look at the Ca^{2+} response itself, Moore et al. [56] have shown that injection of GDP[S] into mouse oocytes inhibits early activation events and that injection of phosducin, which binds to and inhibits G$\beta\gamma$ subunits, partially inhibits oocyte activation [56]. However, Ca^{2+} spiking in response to GTP[S] is inhibited by phorbol esters which, in contrast, have little effect on the Ca^{2+} spikes induced at fertilization [23,27] and this observation has been used as an argument to exclude a role for PLCβ in their generation. The demonstration, in this study, that mouse oocytes have at least two isoforms of PLCβ introduces the possibility that GTP[S] and fertilization may transduce a signal through different combinations of G-protein subunits to separate PLC isoforms, in this case β1 and β3 respectively, and sensitivity to phorbol esters is not an obligatory feature of both pathways. It may thus be premature to preclude a role for G-proteins in mammalian fertilization.

A derivative hypothesis of the receptor/G-protein proposal suggests that Ca^{2+} spiking might be caused by the activation of PLCγ (G-protein-independent). The specific hypothesis is that the sperm binds to a cell-surface receptor which is linked to a tyrosine kinase responsible for activating PLCγ1. Spermatozoa possess, on their external surface, molecules which share sequence similarity to integrins [57]. Recently, it has been shown that the mouse oocyte integrin $\alpha6\beta5$ functions as a sperm receptor [58]. Integrin binding may cause InsP_3 production upon activation of tyrosine kinases coupled to PLCγ [59], a process known to cause Ca^{2+} oscillations in human neutrophils [60]. A CD4/p561ck transducing system very similar to that in T-cells has been found in mouse oocytes [61] and might provide a signal transduction pathway between a receptor linked to a 'soluble' tyrosine kinase and PLCγ. Previous studies in sea urchin eggs have indeed shown that a variety of tyrosine kinases play a role in oocyte activation and that kinase activity increases rapidly in response to sperm binding [62,63]. In *S. purpuratus*, a transmembrane sperm-binding protein has been identified, cloned and sequenced [64,65]. There are five putative cytoplasmic sites for tyrosine phosphorylation which could interact with non-receptor tyrosine kinases [66–69].

Since the stimulation of PLCγ1 in fibroblasts is associated with the phosphorylation of tyrosines 771, 783 and 1254 we attempted to determine whether or not this enzyme was phosphorylated in mouse oocytes following fertilization. There was no evidence of phosphorylation of PLCγ1 linked to sperm binding using either immunoprecipitation or immunodetection of phosphorylated tyrosine residues. However, these experiments were difficult to perform because of the problem of collecting sufficient numbers of oocytes at the appropriate stage of fertilization. Moreover, we do not know whether any sustained phosphorylation is likely to occur or if it is phasic. As an alternative strategy, therefore, we have used different tyrosine kinase inhibitors to test the hypothesis that fertilization of mouse oocytes depends upon tyrosine phosphorylation of PLCγ1. Although somewhat difficult to interpret, there were clear inhibitory effects on spiking and there was also a marked prolongation in the latency between the addition of sperm and the onset of the first spike. While these results seem to support a tyrosine kinase involvement in fertilization these inhibitor studies must be treated with considerable caution not least of all because it is difficult to discern their precise site of action. Inhibition could be at the level of a transmembrane sperm-binding protein, like that described in sea urchin, which might act through a tyrosine kinase signalling cascade. Alteration of its phosphorylation state could affect downstream signalling.

In summary, mouse oocytes contain at least three isoforms of PLC, β1, β3 and γ1, which could participate in a transmembrane signal transduction pathway linking sperm binding to the fertilization Ca^{2+} spikes. These Ca^{2+} spikes appear to be linked to the continuous synthesis of InsP_3 as they cease in the presence of U73122 a PLC inhibitor. As it was not possible to implicate any one of these PLC isoforms in this process, the mechanism by which the spermatozoa induce the Ca^{2+} spikes still eludes us.

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