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Tyrosine Kinase-Dependent Activation of Phospholipase Cγ Is Required for Calcium Transient in *Xenopus* Egg Fertilization

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In a previous study (K.-I. Sato *et al.*, 1999, *Dev. Biol.* 209, 308–320), we presented evidence that a Src-related protein-tyrosine kinase (PTK), named Xyk, may act upstream of the calcium release in fertilization of the *Xenopus* egg. In the present study, we examined whether PTK activation of phospholipase Cγ (PLCγ) plays a role in the fertilization-induced calcium signaling. Immunoprecipitation studies show that *Xenopus* egg PLCγ is tyrosine phosphorylated and activated within a few minutes after fertilization but not after A23187-induced egg activation. Consistently, we observed a fertilization-induced association of PLCγ with Xyk activity that was not seen in A23187-activated eggs. A Src-specific PTK inhibitor, PP1, blocked effectively the fertilization-induced association of PLCγ with Xyk activity and up-regulation of PLCγ, when microinjected into the egg. In addition, a PLC inhibitor, U-73122, inhibited sperm-induced inositol 1,4,5-trisphosphate production and the calcium transient and subsequent calcium-dependent events such as cortical contraction, elevation of fertilization envelope, and tyrosine dephosphorylation of p42 MAP kinase, all of which were also inhibited by PP1. On the other hand, A23187 could cause the calcium response and calcium-dependent events in eggs injected with PP1 or U-73122. These results support the idea that *Xenopus* egg fertilization requires Src-family PTK-dependent PLCγ activity that acts upstream of the calcium-dependent signaling pathway.

Key Words: **protein-tyrosine phosphorylation; protein-tyrosine kinase; phospholipase Cγ; fertilization; egg activation; *Xenopus;* calcium transient; signal transduction.**

INTRODUCTION

Protein phosphorylation and dephosphorylation within cells play a crucial role in regulating cell growth, differentiation, survival, and malignant transformation. In particular, protein-tyrosine phosphorylation catalyzed by protein-tyrosine kinases (PTKs)[2] acts immediately downstream of cell surface receptors that become activated in response to extracellular stimuli (Hunter, 1998). In general, the initial PTK-activating event is transient (from seconds to minutes) and regional (limited to the plasma membrane and submembranous area); however, subsequent PTK-dependent events have a wide variety of consequences. These include up- or down-regulation of biochemical events such as membrane lipid turnover, protein synthesis, and gene transcription, all of which contribute to cellular responses such as cytoskeletal reorganization and cell cycle progression. Thus considerable effort has been made to understand when, where, and how PTKs are regulated and what role PTKs play in cells (Abram and Courtneidge, 2000; Brown and Cooper, 1996; Thomas and Brugge, 1997; Ullrich and Schlessinger, 1990; van der Geer *et al.*, 1994), although function of PTK in the activation of animal development, i.e., fertilization, remains to established.

Egg activation refers to dramatic and dynamic changes in

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[2] Abbreviations used: PTK, protein-tyrosine kinase; IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular calcium level; Xyk, p57 Src-related *Xenopus* tyrosine kinase; PLC, phospholipase; APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; DB, DeBoer's solution; DMSO, dimethyl sulfoxide; PI 4,5-P_2, phosphatidylinositol 4,5-bisphosphate; SB, Steinberg solution; T-TBS, Tris-buffered saline containing Tween 20.

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the egg after its interaction with sperm (Longo, 1997; Yanagimachi, 1994). The fertilizing sperm initiates a series of early and late egg activation events. The early phase of egg activation involves the establishment of a polyspermy block by way of, in most but not all animals so far examined, membrane depolarization, cortical granule exocytosis, and formation of the fertilization envelope. The late phase includes resumption of the cell cycle that is paused at a species-specific stage in the unfertilized egg; this involves pronuclear formation and migration and initiation of protein and DNA synthesis. An inositol 1,4,5-trisphosphate (IP_3)-induced transient increase in intracellular calcium level ($[Ca^{2+}]_i$) (calcium transient) that occurs soon after fertilization has a crucial role for sperm-induced egg activation of almost all species examined (Stricker, 1999). Much recent work has been directed at understanding the molecular mechanism by which the sperm triggers an IP_3-induced calcium transient in the fertilized egg.

There are two major hypotheses about the first messenger that initiates the calcium transient upon fertilization (Foltz and Shilling, 1993; Jaffe, 1996; Nuccitelli, 1991; Swann and Parrington, 1999). One hypothesis is that cytoplasmic or membrane-bound substances that the sperm delivers via its fusion with egg plasma membrane initiate the calcium transient. Alternatively, the calcium transient might be initiated by an egg plasma membrane receptor(s) for sperm. It should be noted that these two hypotheses are not mutually exclusive.

In the African clawed frog, *Xenopus laevis,* the idea of receptor-mediated egg activation has been supported by the following observations: (1) synthetic peptides with a disintegrin motif such as RGD (Iwao and Fujimura, 1996) and KTC (Shilling *et al.,* 1998) can cause egg activation responses and (2) a sperm protease with specificity similar to cathepsin B is capable of activating the egg (Mizote *et al.,* 1999). The former observation suggests that a disintegrin–integrin interaction, which has also been implicated in mammalian gamete interaction (Myles and Primakoff, 1997; Snell and White, 1996), acts in initiating a calcium transient, as seen in T cells and fibroblasts (Kanner *et al.,* 1993; Schwartz *et al.,* 1995). The latter suggests that proteolytic activation of an egg receptor results in the calcium transient, as seen in the activation of the thrombin receptor (Schmaier, 1998) and the urokinase plasminogen activator receptor (Ghiso *et al.,* 1999; Schmaier, 1998) in platelets and monocytes, respectively. These cell surface receptor-mediated calcium responses often involve G proteins and/or PTKs within the cells.

The involvement of a G protein or PTK in *Xenopus* fertilization was initially suggested by findings that ectopically expressed and ligand-stimulated serotonin receptors (Kline *et al.,* 1988) or epidermal growth factor receptors (Yim *et al.,* 1994) could cause egg activation responses. Endogenous G proteins of the pertussis toxin-insensitive G_q class function in *Xenopus* egg activation by the serotonin receptor (Kline *et al.,* 1991). But, Runft *et al.* (1999) have shown that inhibition of G_q did not affect the sperm-induced egg activation.

On the other hand, evidence is accumulating for the involvement of a PTK in *Xenopus* fertilization (for review see Sato *et al.,* 2000). Fertilization of *Xenopus* eggs is accompanied by a transient increase in tyrosine phosphorylation of egg proteins (Sato *et al.,* 1998) and several PTK-specific inhibitors have been shown to inhibit egg activation (Glahn *et al.,* 1999; Sato *et al.,* 1998, 1999). Importantly, the block of egg activation by PTK inhibitors could be overcome by the calcium ionophore A23187. These results suggest that egg PTK(s) may be activated immediately after fertilization and play a crucial role in mediating the calcium transient. In this connection, we have recently identified a Src-related PTK that is activated within 1 min after fertilization (Sato *et al.,* 1996). Pharmacological and parthenogenetic egg activation experiments have demonstrated that the egg PTK, named Xyk (p57 Src-related *Xenopus* tyrosine kinase), may act upstream of calcium-dependent signaling in fertilization (Sato *et al.,* 1999).

Because of the role of PTK in calcium release, it is logical to consider involvement of phospholipase C (PLC) in *Xenopus* egg fertilization. The PLCs constitute a family of enzymes which generate IP_3 and diacylglycerol from phosphatidylinositol 4,5-bisphosphates and include three isozymes: β, γ, and δ (Rhee and Bae, 1997; Singer *et al.,* 1997). In general, PLCβ is up-regulated by receptor-coupled heterotrimeric G proteins while PLCγ is up-regulated by receptor or cytoplasmic PTKs; the activation mechanism of PLCδ is less clear. In *Xenopus* eggs, genes encoding one β (Accession No. L20816) and two γ isozymes (partial cDNAs, Accession Nos. AF090111 and AF090112) have been cloned and their protein products have been detected in eggs (Filtz *et al.,* 1996; Runft *et al.,* 1999). Until now, however, there is no report about whether fertilization stimulates PLC activity in the *Xenopus* egg.

In this paper, we present data that PLCγ becomes tyrosine phosphorylated and is activated as early as a few minutes after fertilization of *Xenopus* eggs. Results obtained with the use of specific inhibitors are consistent with the idea that an egg Src family PTK-dependent PLCγ pathway is required for sperm-induced activation of *Xenopus* eggs. Its physiological importance is discussed in relation to other model organisms.

MATERIALS AND METHODS

Frogs, Gametes, Chemicals, and Antibodies

Wild type and albino African clawed frogs, *X. laevis,* were purchased from Hamamatsu Seibutsu Kyozai (Hamamatsu, Japan). Frogs were maintained in polypropylene tanks filled with dechlorinated tap water or deionized water (1–1.5 liters per animal) at 18–21°C with feeding twice a week. The following procedures were

also carried out at 18–21°C. Three to four days prior to the experiment, female frogs were injected with 40 IU/animal of pregnant mare serum gonadotropin (PMSG; Seikagaku Kogyo, Tokyo). To stimulate ovulation, 250–500 IU/animal of human chorionic gonadotropin (Teikoku Zoki, Tokyo) was injected into the PMSG-injected frogs the night before the experiment. Generally, ovulation started 6–8 h after the second hormonal injection. At this time, the frogs were transferred to a new tank filled with fresh deionized water supplemented with 100 mM NaCl. Ovulated or gently squeezed eggs were dropped into 100-mm plastic dishes filled with 1× DeBoer's solution (DB), pH 7.2, containing 110 mM NaCl, 1.3 mM KCl, and 0.44 mM $CaCl_2$. Eggs were used within 3 h after ovulation (see below). Testes were removed surgically from male frogs that had been injected with 40 IU/animal of PMSG 1–2 days before the experiment and then sacrificed. Testes were kept intact at 4°C in a microfuge tube filled with 1× DB supplemented with 3 mg/ml bovine serum albumin and used within 1 week (see below).

A23187 was obtained from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Protease inhibitors leupeptin and (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF) were purchased from Peptide Institute (Osaka) and Wako Pure Chemicals (Osaka), respectively, dissolved in water at 100 mg/ml and 100 mM, respectively, and kept at −30°C until use. [γ-^{32}P]ATP (No. 35020) was obtained from ICN (Costa Mesa, CA). [^{3}H]Phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) was from NEN Life Science Products (Boston, MA). Bovine brain PI 4,5-P_2 was from Sigma. A synthetic tyrosine kinase substrate peptide, Cdc2 peptide (residues 7 to 26 of fission yeast Cdc2 protein: Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys-Ala-Arg-His-Lys-Leu-Ser), was synthesized and purified as described previously (Fukami *et al.*, 1993). A tyrosine kinase inhibitor, PP1, and its inactive analogue PP3 were obtained from Calbiochem (San Diego, CA). A PLC inhibitor, U-73122, and its inactive analogue U-73343 were from Research Biochemicals International (Natick, MA). All these inhibitors were dissolved in DMSO at 5 mM and kept at −80°C until use.

A mixed mouse monoclonal antibody and a rabbit polyclonal antibody against mammalian PLCγ were from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against either MAP kinase or phosphorylated MAP kinase (anti-phospho MAPK) were from BioLabs (Beverly, MA). Mouse monoclonal anti-phosphotyrosine antibodies 4G10, PY20, and PY99 were purchased from Upstate Biotechnology, ICN, and Santa Cruz (Santa Cruz, CA), respectively. L-Phosphotyrosine, L-phosphoserine, and L-phosphothreonine were obtained from Sigma. A rabbit polyclonal antibody, anti-pepY (anti-Xyk) antibody, was raised against a synthetic peptide that corresponds to residues 410 to 428 of the chicken c-Src protein and was shown to recognize p57 Xyk as described previously (Sato *et al.*, 1996, 1999). Protein A–Sepharose CL-4B was from Amersham Pharmacia (UK). Unless otherwise indicated, other chemicals used were from Sigma, Wako, or Nacalai (Kyoto).

Egg Activation and Egg Extract

Before activation treatment, egg jelly was removed by gentle swirling in 1× DB supplemented with 2% cysteine and 0.06 N NaOH, pH 7.8, for 3 to 8 min. After the dejellying treatment, cysteine was removed by washing five times with 0.5× Steinberg solution (SB) containing 29 mM NaCl, 0.34 mM KCl, 0.17 mM $CaCl_2$, 0.43 mM Mg_2SO_4, and 2.3 mM Tris–HCl, pH 7.4. Egg jelly water was prepared by gentle rocking (50 rpm) of jelly-intact eggs (5 g) in a 60-mm plastic culture dish filled with 10 ml of 1/3 × modified Ringer's solution containing 33.3 mM NaCl, 0.6 mM KCl, 0.33 mM $MgCl_2$, 0.66 mM $CaCl_2$, 1.67 mM Hepes–NaOH, pH 7.8. After 60 min of rocking, soluble material was collected and Ficoll (MW 400,000; Sigma) was added to give a final concentration of 10% (w/v). The Ficoll-supplemented egg jelly water was stored in aliquots at −30°C and used within 2 months after preparation.

Groups of 20 dejellied eggs were then placed in a plastic culture dish (35-mm diameter) filled with fresh 0.5× SB. For fertilization, a piece of testis was macerated in 1 ml of egg jelly water. The suspension of sperm and egg jelly water (100 μl) was applied near the top surface of the eggs. For parthenogenetic egg activation with calcium ionophore, dejellied eggs maintained in 0.5× SB were transferred to new dish filled with fresh 0.5× SB supplemented with 0.2 μM A23187. At the specified time after activation treatment (application of either sperm or A23187), eggs were washed three times with 0.5× SB, immediately frozen by liquid nitrogen, and stored at −80°C. This procedure takes about 30 s between the start of the wash treatment and the freezing. Only batches of eggs that showed successful egg activation rate of more than 80% within 20 min of the activation treatment were used for experiments. The successful egg activation rate was determined by monitoring the occurrence of cortical contraction of the pigmented area—a hallmark of successful egg activation—in another group of the same batch of eggs. By using this procedure, successful egg activation rates as a function of insemination time were scored as 41% in 1 min (number of eggs activated/tested: 16/39), 49% in 2 min (17/35), 73% in 5 min (29/40), 84% in 10 min (31/37), 86% in 15 min (36/42), and 97% in 20 min (38/39) (Fig. 1A). This might reflect the relatively slow time course of PLCγ up-regulation (Fig. 1A), PLCγ-Xyk association (Fig. 2A), and calcium transient (Fig. 5) in response to fertilization. On the other hand, the egg activation scores as a function of time of A23187 treatment were 65% in 1 min (number of eggs activated/tested: 13/20), 73% in 2 min (19/26), 90% in 5 min (18/20), 100% in 10 min (17/17), 100% in 15 min (19/19), and 100% in 20 min (15/15) (Fig. 1A).

For biochemical and immunochemical studies (see below), frozen eggs prepared as above were mixed with 20 μl/egg of ice-cold Triton X-100-containing buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF). The mixture was vortex-mixed and sonicated for 2 min with a TOMY UD-201 ultrasonic disrupter (Tomy Seiko, Tokyo). The sample was then centrifuged at 15,000 rpm for 10 min. The resultant supernatant was carefully taken and stored as Triton X-100-solubilized egg extract. The egg extract was then subjected to further analysis within 2 h of the extraction. In general, 25–32 μg of protein was recovered from one egg. Protein in egg extract was determined spectrophotometrically with the use of a protein assay kit (Bio-Rad, Richmond, CA). Calibration was made with a bovine serum albumin standard (Calbiochem). When IP_3 was assayed, an alternative method was applied to obtain egg extract (see below).

Microinjection

In order to determine a requirement of egg PTK and PLC activity for *Xenopus* egg activation, we performed microinjection of the unfertilized eggs with a specific PTK or PLC inhibitor. Inhibitors used were PP1, a PTK inhibitor which has a strong specificity to the

Src family enzymes (Hanke *et al.*, 1996), and U-73122, a potent PLC-specific compound with broad specificity toward all PLCs (Smith *et al.*, 1990). We also employed PP3 and U-73343, each of which is a structurally related but inactive analogue of PP1 and U-73122, respectively, as a negative control. When required, the stock solutions (5 mM in DMSO) were diluted with buffer containing 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, and an appropriate amount of DMSO so that the final concentration of DMSO in all the diluted solutions was 5%. The inhibitor solutions were then loaded into an oil-filled glass capillary (Drummond), with a tip diameter of 10–30 μm, that was connected to a pulse-directed injector system (Nanoject; Drummond). Before being microinjected, dejellied eggs were washed and incubated with injection buffer containing 110 mM NaCl, 1.3 mM KCl, 20 mM $MgSO_4$, 0.1 mM EGTA, and 10 mM chlorobutanol. Quantitative injection of the inhibitor solution (50 nl per egg) was made under microscopic observation. The cytoplasmic volume of the *Xenopus* egg has been estimated to be about 1 μl, that is, 20 times larger than the buffer volume injected into eggs. Therefore, the final concentration of the inhibitor in the egg was estimated to be 0.1, 0.3, 1, 3, and 10 μM, upon microinjection of the inhibitor solutions of 2, 6, 20, 60, and 200 μM, respectively. After the injection, eggs were healed in 1× DB for 10–15 min. During this time, any prick-activated or dead eggs, which show cortical contraction or abnormal white-coloring, were removed. Then, eggs were washed three times with 0.5× SB and subjected to egg activation treatment (see above).

Immunoprecipitation

For immunoprecipitation, protein samples (Triton X-100-solubilized egg extract, see above) (100–500 μg protein) were incubated with the specified amount of antibody for 3–5 h at 4°C. Immune complexes were collected by adsorption onto protein A–Sepharose. To remove nonspecifically bound materials, the Sepharose beads were washed three times with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF). The washed beads were then used for further analysis (see below). When secondary immunoprecipitation was conducted (Fig. 2B), immune complexes on the beads were dissociated by boiling for 3 min in the presence of 0.5% SDS. After SDS treatment, proteins were recovered by brief centrifugation (10,000 rpm, 1 min), cooled on ice, and mixed with 10-fold volume of 20 mM sodium phosphate, pH 7.5, containing 5 μg of either anti-pepY IgG or preimmune IgG. The mixture was incubated at 4°C overnight and immune complexes were collected as described above. By the immunoprecipitation and immunoblotting methods (see below) with the use of anti-mammalian PLCγ antibody, we have detected a 145-kDa PLCγ protein, whose molecular size matches exactly with that of mouse PLCγ on SDS–PAGE (data not shown), in Triton X-100-solubilized extract of *Xenopus* eggs (Fig. 1A).

Immunoblotting

Protein samples (Triton X-100-solubilized egg extracts with or without immunoprecipitation) were mixed with a concentrated SDS sample buffer (Laemmli, 1970) and boiled for 3 min. The SDS-treated proteins were separated by SDS–PAGE using 8% polyacrylamide gels and transferred to polyvinylidene difluoride membranes using a semidry blotting apparatus (Bio-Rad). Membranes were blocked with T-TBS buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 supplemented with 3 mg/ml bovine serum albumin for 1 h and then incubated for 2–4 h with a primary rabbit or mouse antibody as specified in the text. After the primary antibody treatment, the membranes were washed with T-TBS and then incubated for 1 h with alkaline phosphatase-conjugated goat polyclonal anti-rabbit IgG antibody (Cappel, Belgium) at a dilution of 1:1000. When mouse antibody was used as the primary antibody, the membranes were treated for 1 h with rabbit polyclonal anti-mouse IgG antiserum (Cappel) at a 500-fold dilution before the treatment with the enzyme-conjugated antibody. Immune complexes were visualized by incubating the membranes with buffer containing 0.1 M Tris–HCl, pH 9.5, 5 mM $MgCl_2$, 0.1 M NaCl, 50 μg/ml 5-blomo-4-chloro-3-indolyl phosphate *p*-toluidine salt, and 150 μg/ml nitro blue tetrazolium. The color development proceeded for 15 min and was terminated by washing of the membranes with deionized water. Specificity of the anti-phosphotyrosine immunoblotting data (Figs. 1A and 4A) was verified by competition studies with the use of phosphoamino acids. Inclusion of 10 mM L-phosphotyrosine at the primary antibody treatment completely eliminated the signal, whereas neither 10 mM L-phosphoserine nor 10 mM L-phosphothreonine showed such effect (data not shown).

PLC Assay

Immunoprecipitated protein samples (10 μl of beads) were washed two times with PLC assay buffer containing 50 mM Tris–HCl, pH 7.5, 70 mM KCl, 1 mM $CaCl_2$, 1 mM EGTA, and 0.2% β-octylglucoside. The PLC reaction was initiated by adding 40 μl of PLC assay buffer supplemented with mixed micelles of phosphatidylethanolamine and [^{3}H]PI 4,5-P_2 (148 Bq/nmol) at final concentrations of 100 and 200 μM, respectively. After 15-min incubation at 30°C, the reaction mixture was separated from beads by a brief centrifugation (10,000 rpm, 1 min) and added to 100 μl of water containing 10 μg of bovine serum albumin and 100 nmol of unlabeled PI 4,5-P_2. The mixture was then immediately combined with 250 μl of ice-cold 10% trichloroacetic acid. The mixture was centrifuged at 15,000 rpm for 10 min at 0°C. The resultant supernatant was collected and centrifuged again. The final supernatant (ca. 400 μl) was mixed with 5 ml of a scintillation cocktail (Dojindo, Tokyo) and subjected to β counting with the use of a liquid scintillation counter (Beckman LSC6000).

Protein/Peptide Kinase Assay

Immunoprecipitated protein samples (10 μl of beads) were washed two times with kinase assay buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM dithiothreitol. The kinase reaction was initiated by incubating the washed beads with the kinase assay buffer (15 μl) supplemented with [γ-^{32}P]ATP (3.7 kBq/pmol) at a final concentration of 2 μM. When required, Cdc2 peptide (final concentration of 1 mM) was included in the kinase reaction as an exogenous tyrosine kinase substrate. In Fig. 3A, purified Xyk (Sato *et al.*, 1996) was used as an enzyme source. The reaction proceeded for 10 min at 30°C and was terminated by the addition of SDS-sample buffer followed by boiling for 3 min. Phosphorylated proteins and peptides were separated by SDS–PAGE using 8 and 16% polyacrylamide gels, respectively. Positions of Cdc2 peptide were located by staining of gels with Coomassie brilliant blue. ^{32}P-labeled proteins and Cdc2 peptide were visual-

ized and quantified by BAS2000 bioimaging analyzer (Fujifilm, Tokyo).

IP_3 Extraction and Assay

All the manipulations were carried out on ice or at 4°C. Samples in groups of 10 eggs were homogenized with a glass pestle in 120 μl of 15% (v/v) trichloroacetic acid. The insoluble materials were precipitated by centrifugation at 5000 rpm for 15 min. The resultant supernatants were extracted three times with 10 volumes of water-saturated diethyl ether and then neutralized with 1 M $NaHCO_3$ (10 μl per 100-μl extract). IP_3 in the neutralized extracts was measured with the use of a D-*myo*-IP_3 [^{3}H] assay system (Amersham Pharmacia). This assay was based on the competition between unlabeled IP_3 in the sample and a fixed quantity of a tracer, [^{3}H]IP_3, for a limited number of binding sites on IP_3-specific binding protein preparation supplied by the kit. In our experiments, the neutralized extracts of 100 μl (about 80% of the total; 10 eggs) were subjected to IP_3 assay according to the manufacturer's instruction, thus the calculation of the data involved the dilution factor of 100/80 × 1/10 (total egg number tested in one trial) to determine the amount of IP_3 in one egg. Calibration of IP_3 (from 0.19 to 25 pmol per assay) was made by using the same kit on the same day of experiment.

Intracellular Calcium Release Assay

For calcium measurement, dejellied albino eggs were comicroinjected with 50 nl of 160 μM fura-2 (Molecular Probes, Eugene, OR), a fluorescent calcium indicator, plus 200 μM inhibitor (see "Microinjection"). The injected eggs were then treated with sperm or A23187 and fluorescent signal was recorded by ratio-imaging microscopy using a high-frame digital CCD imaging ARGUS/HISCA system (Hamamatsu Photonics, Japan) with a 10× planneofluar objective, NA 0.3 (Zeiss, Germany). Excitation wavelengths of 340 and 380 nm (each irradiation constant was set to about 800 ms) were used while monitoring the emission at 510 nm. All data collections were made at 10-s intervals at 18–21°C.

RESULTS

Transient Tyrosine Phosphorylation and Activation of PLCγ in Fertilized Xenopus Eggs

We first asked whether *Xenopus* egg PLCγ is tyrosine phosphorylated and activated upon fertilization. In Fig. 1A, Triton X-100-solubilized extracts were prepared from eggs treated with either sperm (insemination) or A23187 for the indicated time. Protein samples were subjected to immunoprecipitation with anti-PLCγ antibody followed by immunoblotting with either anti-phosphotyrosine antibody (top) or anti-PLCγ antibody (bottom). Tyrosine phosphorylation of PLCγ increased as early as 1 min after insemination (upper left). It peaked at 5 to 10 min postfertilization and declined thereafter. The anti-phosphotyrosine signal was specific because the addition of phosphotyrosine, but neither phosphoserine nor phosphothreonine, could eliminate the signal (see Materials and Methods). Increased tyrosine phosphorylation of PLCγ was not detected during A23187-induced egg activation (upper right). Under both egg activation conditions, coimmunoprecipitation of any other tyrosine phosphorylated proteins could not be convincingly detected. Immunoblotting with anti-PLCγ antibody revealed that the relative amount of PLCγ in the anti-PLCγ immunoprecipitates was not changed (Fig. 1A, bottom). We concluded therefore that PLCγ is transiently tyrosine phosphorylated in fertilized eggs and that the increase in $[Ca^{2+}]_i$ is not sufficient to mimic this event. We should note that using our fertilization procedures, egg activation after insemination was somewhat asynchronous, reaching 50% after a 2-min insemination period (Fig. 1A, see % egg activation). This may account for the relatively slow peak response of fertilization-induced tyrosine phosphorylation of PLCγ.

To assess whether tyrosine phosphorylation of PLCγ correlates with up-regulation of its enzymatic activity, the anti-PLCγ immunoprecipitates prepared as above were subjected to an *in vitro* PLC assay with [^{3}H]PI 4,5-P_2 as a substrate. As shown in Fig. 1B, fertilization resulted in a transient increase (two- to threefold) in PLCγ activity. A23187-activated eggs did not show this increase; the small but significant decrease in PLCγ activity was observed. The data were somewhat surprising because in mammal cells, PLCγ can be activated by increasing $[Ca^{2+}]_i$. This might reflect that negative feedback of PLCγ activity is taking place in response to the A23187 treatment of eggs. The peak response of PLCγ enzymatic activation in fertilized eggs was somewhat slower than is usually seen in growth factor-stimulated mammalian cells (within 1 min). Again, this might be due to the time required for successful sperm–egg interaction (Fig. 1A, see % egg activation).

Transient Association of PLCγ with an Egg PTK (Xyk) upon Fertilization

As described in the Introduction, we have recently found that a 57-kDa Src-related egg PTK, named Xyk, is activated following a 1-min insemination. (*Note:* In this case, we have used jelly-intact eggs. Therefore, an additional 3–6 min for dejelly treatment and wash treatment is necessary before egg extraction.) The results obtained in Fig. 1 led us to examine whether Xyk is in close contact with PLCγ in eggs. In Fig. 2A, Triton X-100-solubilized egg extracts were prepared from unfertilized, fertilized, and A23187-activated eggs as in Fig. 1, immunoprecipitated with anti-PLCγ antibody, and the immunoprecipitates were subjected to a protein kinase assay without (top) or with an exogenous PTK substrate (Cdc2 peptide, bottom). The resultant ^{32}P-labeled proteins and peptide were separated by SDS–PAGE and visualized using a bioimaging analyzer. These experiments showed that an ~60-kDa phosphorylated protein (p60) coimmunoprecipitated with PLCγ, in samples prepared from eggs inseminated for 2, 5, and 10 min (Fig. 2A, top, lanes 3, 4, and 5). We should note that the pattern of coprecipitated and phosphorylated proteins in Fig. 2A was

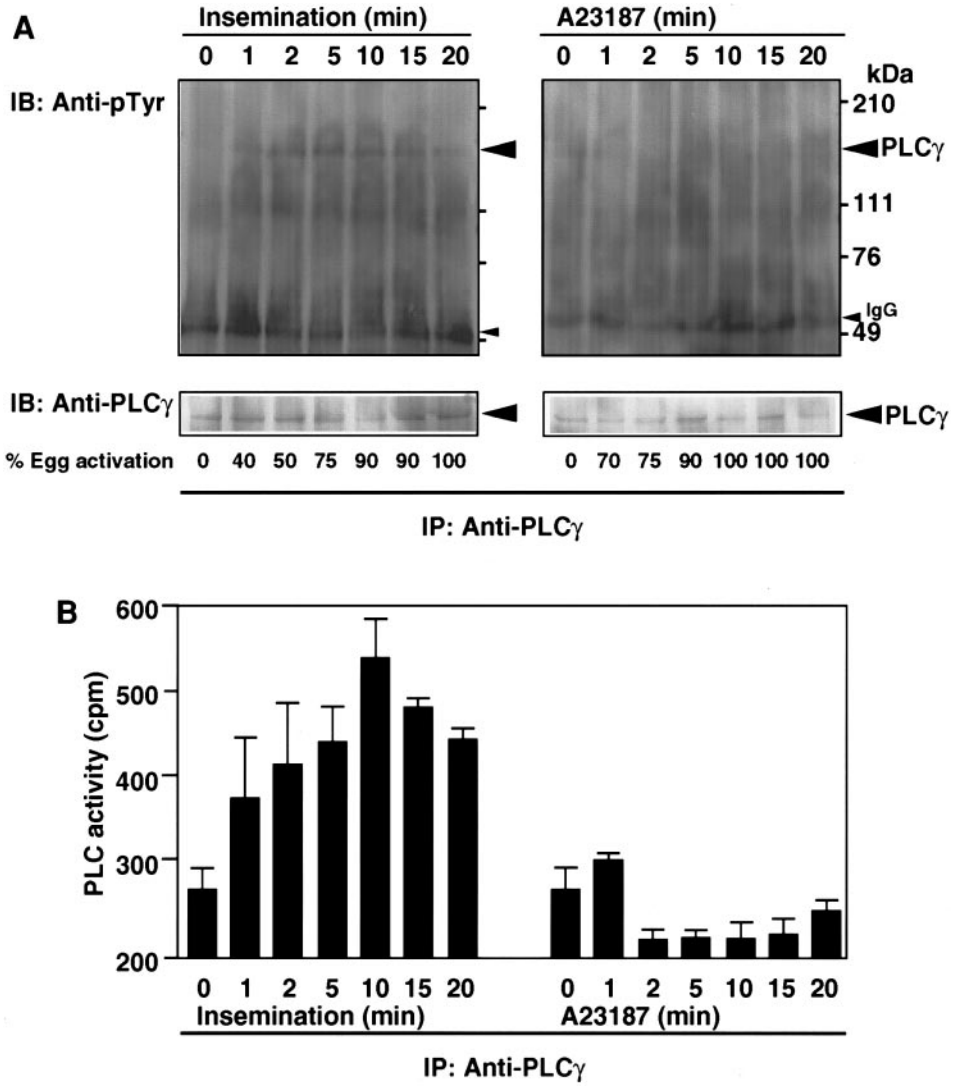

FIG. 1. Tyrosine phosphorylation and activation of *Xenopus* egg PLCγ upon fertilization. Triton X-100-solubilized egg extracts (300 μg protein) were prepared from *Xenopus* eggs that had been untreated (0 min) or treated for the indicated time with either sperm (insemination) or A23187 and immunoprecipitated (IP) with anti-PLCγ antibody. (A) The anti-PLCγ immunoprecipitates were separated by SDS–PAGE and analyzed by immunoblotting (IB) with either anti-phosphotyrosine antibody (top) or anti-PLCγ antibody (bottom). Approximate values of successful egg activation (% egg activation), as judged by the occurrence of cortical contraction (see Materials and Methods for detail), are also indicated. Shown are representative results of three independent experiments. The positions of p145 PLCγ and heavy chains of IgG are indicated by arrowheads. Prestained molecular size markers (Bio-Rad) were myosin (210 kDa), β-galactosidase (111 kDa), bovine serum albumin (76 kDa), and ovalbumin (49 kDa). (B) The anti-PLCγ immunoprecipitates were subjected to a PLC assay using [^{3}H]PI 4,5-P_2 as described under Materials and Methods. The radioactivity of hydrolyzed product, [^{3}H]IP_3, was counted in a liquid scintillation counter. The data represent means ± standard deviations of three independent experiments.

quite different from that in Fig. 1A. This discrepancy might be due to the fact that we used two different methods in these two experiments: Fig. 1A involves immunoblotting, whereas Fig. 2A involves protein kinase assay with ^{32}P-labeled ATP. The Cdc2 peptide substrate was also phosphorylated in these samples (bottom, lanes 3, 4, and 5). The time course of the kinase activity in the immunoprecipitates correlated closely with that of the tyrosine phosphorylation and activation of PLCγ (see Fig. 1). The increase in kinase activity was not observed in response to A23187-induced egg activation (right, lanes 8–14). Several other ^{32}P-labeled proteins with molecular size of 80–150 kDa

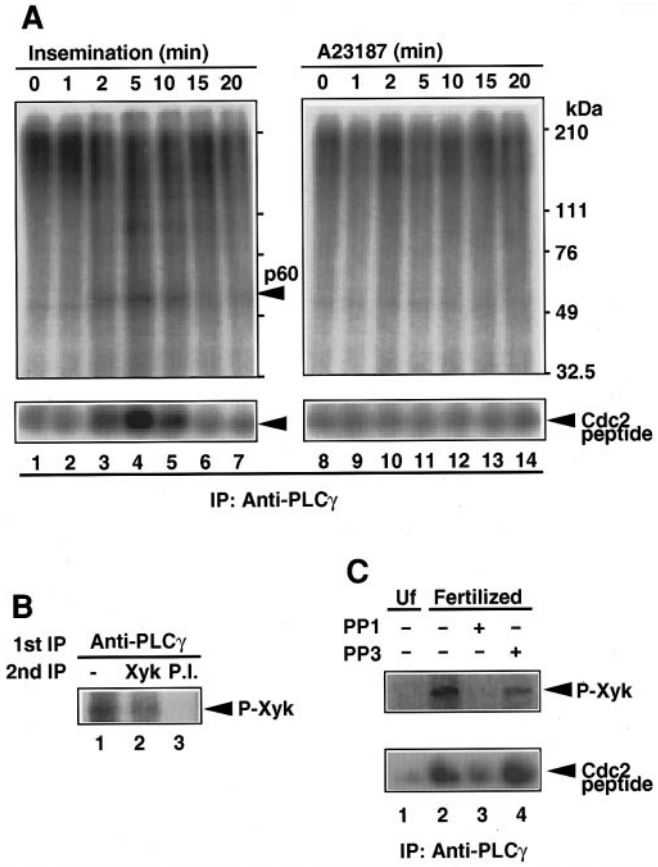

FIG. 2. Transient association of PLCγ with egg PTK Xyk during fertilization. Triton X-100-solubilized egg extracts (300 μg protein per sample) were prepared from eggs that had been treated with either sperm (insemination) or A23187 for the indicated period and immunoprecipitated (IP) with anti-PLCγ antibody as in Fig. 1. (A) The anti-PLCγ immunoprecipitates were subjected to a protein kinase assay without (top) or with Cdc2 peptide (bottom) as described under Materials and Methods. The phosphorylated proteins and Cdc2 peptide were separated by SDS–PAGE and analyzed with a BAS2000 bioimaging analyzer. The positions of phosphorylated 60-kDa protein (p60) and Cdc2 peptide are indicated. (B) The anti-PLCγ immunoprecipitate containing p60 (lane 1) that had been incubated with [γ-^{32}P]ATP as for A (5-min fertilized egg sample) was subjected to secondary immunoprecipitation with either anti-Xyk antibody (lane 2, Xyk) or the preimmune serum (lane 3, P.I.) as described under Materials and Methods. The immunoprecipitates were separated by SDS–PAGE and analyzed by BAS2000 bioimaging analyzer. The position of p60 (P-Xyk) is indicated. (C) Anti-PLCγ immunoprecipitates were prepared as in A from unfertilized eggs (lane 1) or fertilized eggs (5 min after insemination) that had been premicroinjected with vehicle (lane 2), 10 μM PP1 (lane 3), or 10 μM PP3 (lane 4) and subjected to a protein kinase assay without (top) or with Cdc2 peptide (bottom). The presence of phosphorylated Xyk (P-Xyk) and Cdc2 peptide was visualized as in A. All results shown are representative of three independent experiments.

were also found in the anti-PLCγ immunoprecipitates of fertilized egg extracts (Fig. 2A, top). At present, however, their identities are not determined.

To determine if the 60-kDa phosphoprotein was Xyk, the PLCγ immunoprecipitate (Fig. 2A) was denatured by SDS treatment and subjected to a secondary immunoprecipitation with an antibody specific to Xyk. p60 was present in the anti-Xyk immunoprecipitate (Fig. 2B, lane 2, P-Xyk), but not in a control sample using the preimmune antibody (lane 3). The results shown in Fig. 2B suggest that fertilization results in association of the activated Xyk with PLCγ, although we do not know whether the association is direct or indirect (see Discussion).

The results shown in Figs. 2A and 2B led us to examine if egg PTK activity, especially that of Src family kinases (including Xyk), is required for PLCγ activation and subsequent egg activation process in fertilized *Xenopus* eggs. To approach this question, we employed PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) as a Src family PTK-specific inhibitor (Hanke *et al.*, 1996). In Fig. 2C, we examined the effect of PP1 or its inactive analogue PP3 on fertilization-induced association of Xyk and Cdc2 peptide kinase activity with PLCγ. The anti-PLCγ immunoprecipitate was prepared from unfertilized eggs or fertilized eggs (5 min postinsemination) that had been injected with PP1 or PP3 at the final concentration of 10 μM and subjected to a protein kinase assay without (top) or with Cdc2 peptide (bottom) as in Fig. 2A. Data demonstrated that upon injection of PP1, but not PP3, fertilization-induced association of Xyk activity by means of autophosphorylation (P-Xyk) and phosphorylation of Cdc2 peptide was diminished. This result indicates that PP1-sensitive PTK activity is necessary for physical interaction between Xyk and PLCγ.

Inhibition of Xyk by PP1 and Inhibition of Egg PLCγ by U-73122

To verify whether PP1 is inhibitory toward Xyk, we examined the *in vitro* effect of PP1 on the kinase activity of an Xyk preparation that has been purified to near homogeneity by several column chromatographic procedures (Sato *et al.*, 1996). As shown in Fig. 3A, phosphorylation of Cdc2 peptide by the purified Xyk was inhibited by PP1 in a dose-dependent manner, but not by PP3 even at the highest final concentration (10 μM) used in this experiment. The half-inhibition concentration was calculated to be 0.7 ± 0.1 μM (n = 3). Similar dose-dependent inhibition pattern and the half-inhibition value were also obtained when a DEAE column fraction of the Triton X-100-solubilized egg membrane, in which Xyk has been shown to be enriched (Sato *et al.*, 1996, 1999), was used as an enzyme source (data not shown). The half-inhibition concentration of PP1 for Xyk is substantially larger than that reported by Hanke *et al.* (1996), who used the immunoprecipitated Lck (IC_{50}, 0.005 ± 0.001 μM) and Fyn (IC_{50}, 0.006 ± 0.001 μM) as an

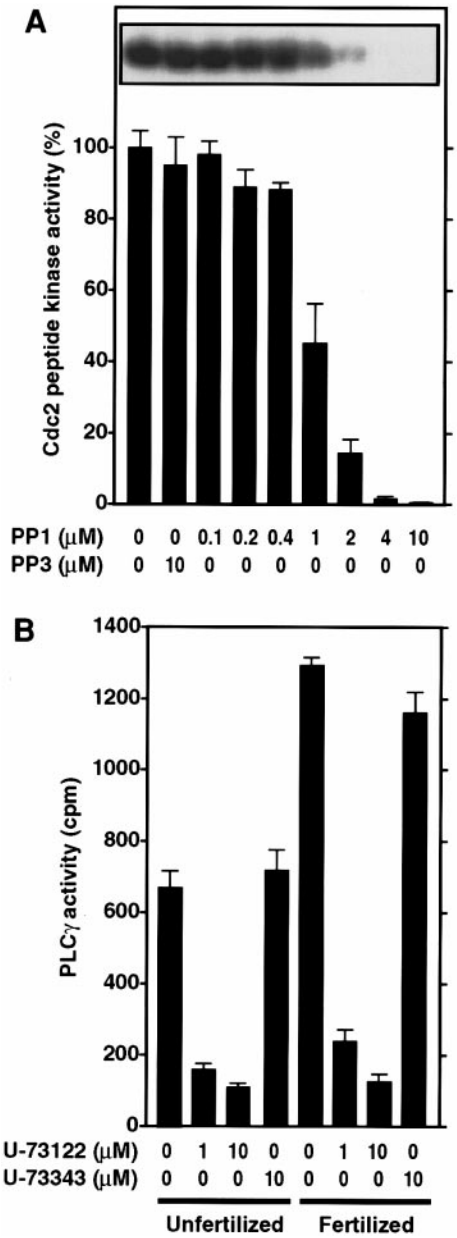

FIG. 3. Dose-dependent and specific inhibition of tyrosine kinase activity of Xyk and PLCγ activity by PP1 and U-73122, respectively. (A) Purified Xyk (200 ng protein) was subjected to a protein kinase assay with 1 mM Cdc2 peptide as a substrate in the absence or presence of various amounts (0.1–10 μM) of PP1 or 10 μM PP3. Phosphorylation of Cdc2 peptide is visualized as in Fig. 2A (shown in inset), quantified, and presented as percentage of control without inhibitor. (B) The anti-PLCγ immunoprecipitates were prepared from unfertilized eggs or fertilized eggs (5 min after insemination) and subjected to a PLC assay in the absence or presence of 1 or 10 μM U-73122 or 10 μM U-73343. The reaction product, $[^3H]IP_3$, was counted in a liquid scintillation counter. The data represent means ± standard deviations of three independent experiments.

enzyme source and enolase as an exogenous substrate. We suggest that the use of different enzyme preparation and substrate protein might reflect the difference in the results.

Recent work has shown that U-73122 (1-[6-[[17β-3-methoxyestra-1,3,5-(10)-trien-17-yl]amino]hexyl]1*H*-pyrrole-2,5-dione) is useful as a potent PLC-specific inhibitor (Lee and Shen, 1998; Smith *et al.*, 1990). As an initial approach with U-73122, we examined the effect of U-73122 on the egg PLCγ activity *in vitro*. In Fig. 3B, the anti-PLCγ immunoprecipitate was prepared from unfertilized eggs or fertilized eggs (5 min postinsemination) as in Fig. 1B and subjected to PLC assay with $[^3H]$PI 4,5-P_2 as a substrate. It is shown that U-73122 at 1 or 10 μM effectively inhibits the PLCγ activity. On the other hand, U-73343, an inactive analogue of U-73122, did not show such inhibitory effect at 10 μM (Fig. 3B). The results demonstrate that egg PLCγ activity obtained with the immunoprecipitation method is sensitive to U-73122 and suggest that this inhibitor may be useful to inhibit egg PLCγ activity *in vivo* by means of microinjection.

Requirement for Src Family PTK Activity in Up-regulation of PLCγ upon Fertilization

To test whether fertilization-induced up-regulation of PLCγ and subsequent egg activation events (see below) involve egg PTK, especially Src family kinases, and egg PLC activity, PP1 or U-73122 was microinjected into *Xenopus* eggs and examined for its ability to block the fertilization-induced biochemical and cell biological events. We usually microinjected the inhibitor as well as its inactive analogue at the final concentration of 10 μM, unless otherwise indicated.

Fertilization-induced tyrosine phosphorylation of PLCγ is significantly reduced in eggs injected with PP1 (Fig. 4A, lane 3), whereas other compounds, PP3 (lane 4), U-73122 (lane 5), and U-73343 (lane 6), did not show such effect. Correspondingly, fertilization-induced enzymatic activation of PLCγ assessed as in Fig. 1B was blocked in eggs injected with PP1, but not in eggs injected with other compounds (Fig. 4B). These results indicate that fertilization-induced tyrosine phosphorylation and activation of PLCγ require the activity of PP1-sensitive egg PTK(s), i.e., Src family PTK(s). The failure of the microinjected U-73122 to block fertilization-induced PLCγ activity might be due to dilution and/or inactivation of the inhibitor during the egg extraction and immunoprecipitation processes. In fact, we have observed that PLCγ activity could be effectively blocked by newly added U-73122 at 10 μM (see Fig. 3B). In addition, we observed that sperm-induced IP_3 production, a measure of *in vivo* PLC activity, could be effectively blocked by U-73122 (see below).

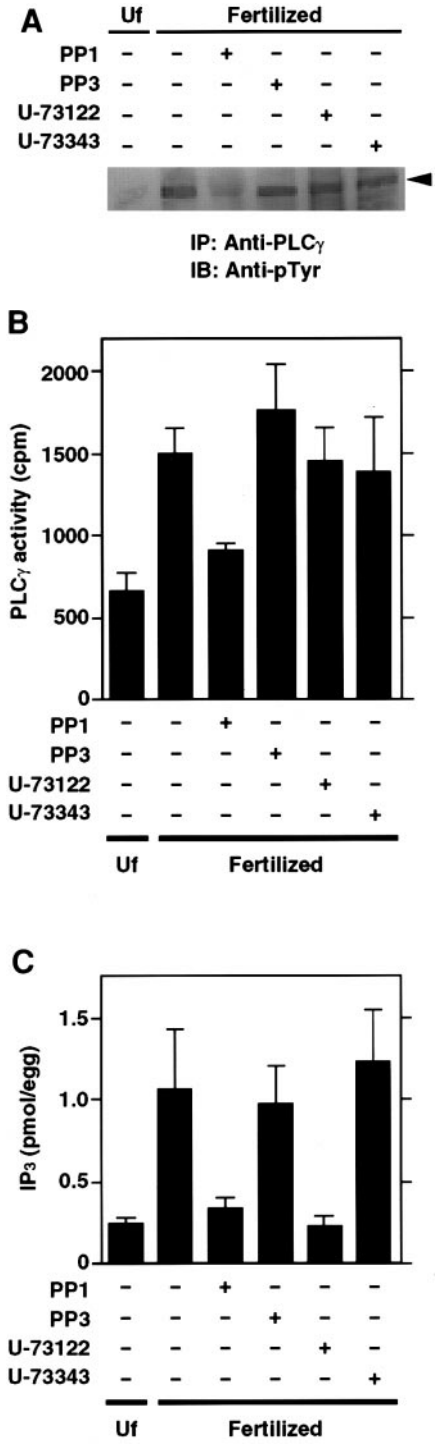

FIG. 4. Effect of the PTK inhibitor PP1 and the PLC inhibitor U-73122 on fertilization-induced stimulation of the PLCγ and IP_3 production. (A and B) *Xenopus* unfertilized eggs were either uninjected (lanes 1 and 2) or injected with the indicated inhibitor (lane 3, PP1; lane 4, PP3; lane 5, U-73122; lane 6, U-73343) at the final cytoplasmic concentration of 10 μM as described under Materials and Methods. Eggs were then inseminated for 5 min (fertilized). Triton X-100-solubilized egg extracts (300 μg protein) were prepared from the fertilized eggs and unfertilized control eggs (Uf) and analyzed for tyrosine phosphorylation of PLCγ (A) and PLCγ activity (B) as in Figs. 1A and 1B, respectively. A depicts a representative result of two independent experiments. The position of tyrosine phosphorylated PLCγ is indicated by an arrowhead. In B, the data represent means ± SD of three independent experiments. (C) *Xenopus* eggs were injected with the indicated inhibitor and inseminated for 5 min (fertilized) as above. Egg samples were extracted with TCA and diethyl ether and IP_3 was determined using the radiolabeled IP_3 assay kit as described under Materials and Methods. The data represent the means ± SD of three independent experiments using 10 eggs each.

Requirement for PTK and PLC Activities in IP_3 Increase and Calcium Transient upon Fertilization

We next examined the effect of PP1 and U-73122 on sperm-induced egg activation. In Fig. 4C, we determined the amount of IP_3 in fertilized eggs injected with each of the inhibitors, by using the radiolabeled IP_3-receptor binding assay system. We estimated IP_3 in unfertilized eggs to be 0.21 ± 0.03 pmol/egg. The value is somewhat larger than those estimated by Stith *et al.* (0.05 pmol/egg; 1994) and by Snow *et al.* (0.13 pmol/egg; 1996). This might be due to the difference in experimental procedures for IP_3 extraction. Importantly, however, we observed that IP_3 increased about 5-fold (up to 1.08 ± 0.44 pmol/egg) 5 min after fertilization (Fig. 4C). This is in agreement with the previous reports showing that fertilization resulted in IP_3 increase with maximum fold of 5.5 (Stith *et al.*, 1994) or 3.2 (Snow *et al.*, 1996). On the other hand, both PP1- and U-73122-injected eggs, but neither PP3- nor U-73343-injected eggs, showed significant reduction in fertilization-induced increase of IP_3. The result clearly demonstrates that IP_3 production in fertilized *Xenopus* eggs require both the Src family PTK activity and the U-73122-sensitive PLC activities. At present, however, we cannot rule out the possibility that sperm-induced IP_3 production may also involve the activity of other PLC isozymes, β and δ, because it is known that U-73122 has broad specificity toward all the isozymes of PLC (Smith *et al.*, 1990; see Discussion).

Since IP_3 plays an indispensable role in the calcium transient upon *Xenopus* egg fertilization (Nuccitelli *et al.*, 1993), we examined whether inhibitor-injected eggs could undergo a calcium transient in response to fertilization, with the use of the calcium-sensitive fluorescent indicator, fura-2. Eggs were co-injected with fura-2 and PP1, PP3, U-73122, or U-73343 and then inseminated, while the fluorescent ratio signal from the eggs was recorded. Figure 5 depicts the representative traces of the calcium measurements and Table 1 summarizes the data obtained in this study. With an inactive inhibitor, PP3 or U-73343, the sperm-induced calcium response was similar to that seen in

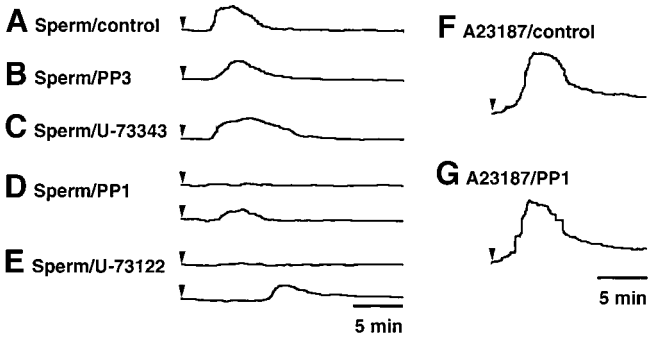

FIG. 5. Inhibition of the fertilization-induced calcium transient by PP1 and U-73122. *Xenopus* albino eggs were co-injected with fura-2 and the indicated inhibitor at final cytoplasmic concentrations of 8 and 10 μM, respectively, and then treated with either sperm (A–E) or A23187 (F and G), as described under Materials and Methods. Shown are representative traces of the fluorescent ratio signal of fura-2 as a function of time. Arrowheads indicate the time at which sperm or A23187 is added. (A) Fura-2-injected control. A calcium transient was detected within 3 min after sperm addition. Eggs injected with PP3 (B) and U-73122 (C) showed similar results. In PP1-injected eggs (D), 86% (19/22) of eggs showed no calcium transient (upper line) while 14% (3/22) of eggs showed a control-like response with reduced peak amplitude (lower line). In U-73122-injected eggs (E), 75% (6/8) of eggs showed no calcium transient (upper line) while 25% (2/8) of eggs showed significant delay in initiating calcium rise (lower line). A23187 immediately induced calcium transient irrespective of the absence (F) or the presence of PP1 (G). A summary of the results is shown in Table 1.

control fura-2-injected eggs (Figs. 5A–5C). Such a normal response in $[Ca^{2+}]_i$ was observed within 3 min after insemination in 94, 100, and 88% of eggs that were injected with fura-2 alone (control), PP3, and U-73343, respectively (Table 1). One of eight U73343-injected eggs and 1 of 17 fura-2 alone-injected (None) eggs did not show the calcium release. This might be due to cell injury and/or calcium buffering effect of fura-2. On the other hand, 87% of PP1-injected eggs and 75% of U-73122-injected eggs showed no calcium response up to 25 min after insemination (Figs. 5D and 5E, each upper line; Table 1). About 14% (3 of 22 eggs) of PP1-injected eggs showed a calcium response in a time course similar to that seen in control eggs, but the peak amplitude was significantly reduced (Fig. 5D, lower line; Table 1). Twenty-five percent (2 of 8 eggs) of U-73122-injected eggs showed a significant delay in initiating calcium response that was normal in the peak amplitude (Fig. 5E, lower line; Table 1). These results indicate that both PP1 and U-73122 are capable of inhibiting the sperm-induced calcium transient in *Xenopus* eggs and suggest that PP1 and U-73122 might act on a distinct step to exert their inhibitory effect. In the case of the A23187-induced calcium transient, however, eggs injected with PP1 (Fig. 5G) showed a response that was indistinguishable from that seen in control-injected eggs (Fig. 5F). A similar response was observed with eggs injected with PP3, U-73122, or U-73343 (Table 1). The results are consistent with the fact that A23187-induced egg activation does not involve up-regulation of both Xyk (Sato *et al.*, 1999) and PLCγ (this study, Fig. 1).

Inhibition of Fertilization-Induced and Calcium-Dependent Cellular Events by PP1 or U-73122

In addition to IP_3/calcium responses, we found that inhibitor-injected eggs showed defects in undergoing several calcium-dependent cytological changes. In Table 2,

TABLE 1

Summary of Ca^{2+} Measurements in *Xenopus* Eggs Injected with Inhibitors and Then Activated by Fertilization or by A23187

	Fertilization			A23187
Inhibitor	% of eggs with Ca^{2+} rise	Time to Ca^{2+} rise (min)	Peak amplitude	% of eggs with Ca^{2+} rise
PP1 (10 μM)	13.6 (22)	3.4 ± 0.2 (3)	1.1 ± 0.3 (3)	100 (4)
PP3 (10 μM)	100 (5)	3.1 ± 0.1	2.0 ± 0.1	100 (4)
U-73122 (10 μM)	25.0 (8)	7.5 (2)	1.7 (2)	100 (4)
U-73343 (10 μM)	87.5 (8)	3.0 ± 0.2 (7)	1.9 ± 0.1 (7)	100 (4)
None	94.4 (18)	3.1 ± 0.1 (17)	2.0 ± 0.2 (17)	100 (5)

Note. Xenopus albino eggs were comicroinjected with fura-2 and the indicated inhibitor at final cytoplasmic concentration of 8 and 10 μM, respectively, and then treated with either sperm (fertilization) or A23187 as described under Materials and Methods. Part of the representative results is shown in Fig. 5. Number of eggs examined, *n*, is indicated in parentheses. Values for the time to Ca^{2+} rise refer to the span between the time of sperm addition and the time at which fluorescent ratio signal versus time trace begins to rise. Values for peak amplitude are presented as the fold increase in the ratio signal over that observed with the unfertilized eggs. Data are represented as the means ± SD. Each data point was obtained with eggs from two to seven animals.

TABLE 2

Effect of PP1 and U-73122 on Sperm-Induced Cytological Changes in *Xenopus* Eggs Treated with Sperm or A23187

Inhibitor concentration (μM)	Fertilization							A23187		
				SEP/egg						
	n	% CC	% FE	0	1	2	3	*n*	% CC	% FE
PP1										
10	25	0	0	15	10	0	0	8	87.5	87.5
3	10	0	0		ND				ND	ND
1	10	50.0	50.0	7	3	0	0	8	75.0	75.0
0.3	14	57.1	64.3		ND				ND	ND
0.1	11	72.7	72.7		ND				ND	ND
PP3										
10	6	66.7	66.7	0	6	0	0	6	100	100
1	10	80.0	100.0	0	10	0	0	8	75.0	75.0
0.1	6	83.3	100.0		ND				ND	ND
U-73122										
10	12	16.7	16.7	1	6	4	1	13	76.9	76.9
3	17	23.5	23.5		ND				ND	ND
1	10	60.0	60.0	0	8	2	0	9	77.8	77.8
0.3	15	73.3	73.3		ND				ND	ND
0.1	10	70.0	70.0		ND				ND	ND
U-73343										
10	19	84.2	78.9	1	18	0	0	14	71.4	71.4
1	11	81.8	81.8		ND			8	87.5	87.5
None	38	92.1	92.1	0	38	0	0	14	100.0	100.0

Note. Eggs were microinjected with the inhibitor at the indicated final cytoplasmic concentration and then activated by sperm or A23187 as described under Materials and Methods. Two egg activation events, cortical contraction (CC) of the pigmented area and elevation of fertilization envelope (FE), were monitored under microscopic observation and scored. In fertilized eggs, we also scored the number of sperm entry point (SEP) (Palecek *et al.,* 1978) in eggs tested. Values of SEP/egg are shown. Number of eggs examined, *n*, is indicated. Each data point was obtained with eggs from three to eight animals. ND, not done.

we summarize the data about the dose-dependent effect of PP1 or U-73122 on cortical contraction (CC) of the pigmented granule and formation of fertilization envelope (FE) in eggs activated by fertilization or A23187. Consistent with the results described above, both PP1 and U-73122 were effective in blocking these cytological processes up to 30 min after fertilization (Table 2). The inhibitory effect was dose-dependent while both PP1 and U-73343 did not show any inhibitory effect on A23187-induced CC and FE up to 10 μM (Table 2). We also analyzed the occurrence of polyspermy in inhibitor-injected eggs, because the polyspermy block in *Xenopus* is entirely dependent on sperm-induced calcium transient (Grey *et al.,* 1982; Kline, 1988; Quill and Hedrick, 1996). As expected, U-73122-injected eggs resulted in polyspermy, as judged by the appearance of multiple sperm entry points (SEP) on the surface of fertilized eggs (Table 2). On the other hand, PP1-injected eggs did not show multiple sperm entry points; a subset of PP1-injected eggs (10 of 25) showed at least one SEP, while we failed to detect even one SEP in the other subset of PP1-injected eggs (15 of 25) (Table 2), suggesting that a very early step in fertilization, i.e., sperm entry, is inhibited in PP1-injected eggs (see Discussion).

We analyzed one more calcium-dependent event in the inhibitor-injected eggs; dephosphorylation of p42 MAP kinase. In unfertilized eggs, p42 MAP kinase is maintained in the tyrosine phosphorylated and active form (Fig. 6, lane 1) and its tyrosine dephosphorylation occurs within 40 min after fertilization (lane 2), as demonstrated by immunoblotting of the egg extract with either anti-phospho MAPK antibody (top) or anti-MAPK antibody (bottom). In eggs injected with either PP1 (lane 3) or U-73122 (lane 5), however, p42 MAP kinase remained tyrosine phosphorylated as in unfertilized eggs. PP3 and U-73343 were consistently inert in blocking dephosphorylation of p42 MAP kinase in fertilized eggs (Fig. 6, lanes 4 and 6). PP1 and U-73122 were again ineffective in A23187-induced dephosphorylation of p42 MAP kinase (data not shown). These data strongly suggest that sperm, but not A23187, requires egg Src family PTK and PLC activity to initiate the egg activation process in *Xenopus*.

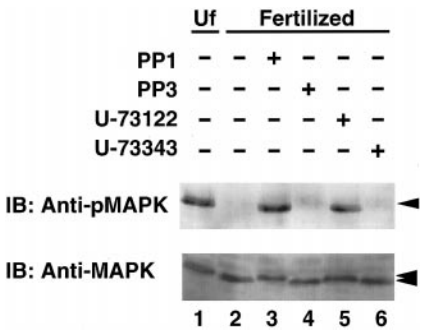

FIG. 6. Inhibition of fertilization-induced tyrosine dephosphorylation of p42 MAPK by PP1 and U-73122. Unfertilized eggs were untreated (Uf) or injected with the indicated inhibitor (each at final cytoplasmic concentration of 10 μM) and then treated with sperm for 40 min (fertilized). Triton X-100-solubilized egg extracts (15 μg protein per sample) were prepared and analyzed by immunoblotting with either an anti-phospho MAP kinase antibody (top) or an anti-MAP kinase antibody (bottom). Shown is a representative result of three independent experiments. An arrowhead in the upper blot indicates the position of tyrosine phosphorylated MAP kinase. In the lower blot, the positions of tyrosine phosphorylated MAP kinase and dephosphorylated MAP kinase are indicated by upper and lower arrowheads, respectively.

DISCUSSION

A role for PLC in sperm-induced egg activation has not been previously described in *Xenopus* but inferred in the studies on other model organisms such as sea urchin (Carroll *et al.*, 1999; Rongish *et al.*, 1999; Shearer *et al.*, 1999), starfish (Carroll *et al.*, 1997; Giusti *et al.*, 1999a), and mouse (Deng *et al.*, 1998; Dupont *et al.*, 1996) (see below). The results obtained in the present study highlight the possibility that there may be a requirement of Src family PTK-dependent PLCγ activity for sperm-induced activation of *Xenopus* egg (Fig. 7).

Requirement of Src Family PTK-Dependent PLC Activity in Xenopus Egg Activation

In this study, we have shown that *Xenopus* egg fertilization is accompanied by up-regulation of egg PLCγ (Fig. 1) that is associated with the Xyk interaction (Fig. 2) and sensitive to PP1 (Figs. 4A and 4B). In addition, both PP1 and U-73122 were shown to inhibit several aspects of sperm-induced egg activation processes: the IP_3 increase (Fig. 4C), calcium transient (Fig. 5, Table 1), cortical contraction, formation of fertilization envelope, successful sperm entry or block of polyspermy (Table 2), and tyrosine dephosphorylation of p42 MAP kinase (Fig. 6). From these results, we suggest that sperm-induced activation of *Xenopus* eggs involves a signaling cascade: Src family PTKs (Xyk) $\rightarrow$ PLC$\gamma \rightarrow IP_3 \rightarrow$ calcium transient $\rightarrow$ egg activation processes (Fig. 7).

PP1 is a highly specific Src kinase inhibitor (Hanke *et al.*, 1996; Liu *et al.*, 1999; Schindler *et al.*, 1999). In *Xenopus* eggs, Xyk is the most prominent Src family member at the protein level and has been shown to be activated in response to fertilization (Sato *et al.*, 1999). So, we believe that a large part of PP1′s inhibitory effect on egg activation is due to the inhibition of Xyk activity. In accordance with this, it is demonstrated that Xyk activity can be effectively blocked by PP1 *in vitro* (Fig. 3A). At the moment, however, we cannot rule out the possibility that PP1 also affects the activity of other unidentified PTKs within eggs, especially because PP1 at 10 μM will have potency to inhibit non-Src family PTKs such as receptor types (Hanke *et al.*, 1996). To clarify this issue, it will be necessary to examine whether PP1-sensitive egg PTKs, other than Xyk, are present in *Xenopus* eggs.

Use of PP1 has recently been described in a fertilization

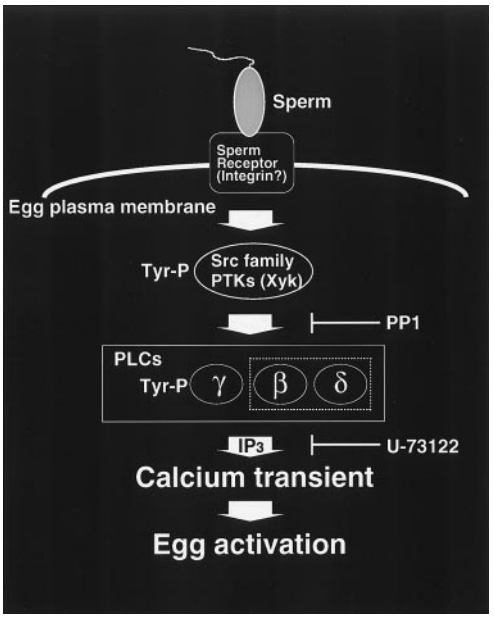

FIG. 7. A model for the sperm-induced activation of *Xenopus* egg that involves PTK-dependent PLCγ activity. *Xenopus* sperm–egg interaction that seems to be mediated by sperm disintegrin and egg integrin results in the onset of intracellular fertilization signaling which involves tyrosine phosphorylation and activation of Src-family PTKs. One of the Src-related PTK, Xyk, was shown to interact with, phosphorylate, and activate PLCγ indirectly or directly, in a PP1-sensitive manner. The activated PLCγ may be responsible for IP_3-induced calcium transient and subsequent egg activation because U-73122 can block effectively these events, although the involvement of other PLC isozymes, β and δ, is uncertain. Since the inhibition of egg activation by PP1 and U-73122 can be overcome by application of the calcium ionophore A23187, the targets of the inhibitors, e.g., Src-family PTK (Xyk) and PLCs, must act upstream of the calcium transient in the signal transduction pathway of *Xenopus* egg fertilization.

study of sea urchin eggs (Abassi *et al.*, 2000). In this species, PP1 has been shown to cause delay in the onset of calcium release from the endoplasmic reticulum at fertilization. In addition, there are some reports concerning the effect of other PTK inhibitors in *Xenopus* as well as in other organisms. In *Xenopus*, we have recently observed that eggs preincubated with either genistein or herbimycin A do not undergo cortical contraction and tyrosine dephosphorylation of MAP kinase in response to fertilization (Sato *et al.*, 1998). Glahn *et al.* (1999) reported that *Xenopus* eggs injected with other PTK inhibitors such as lavendustin A and peptide A, a synthetic peptide inhibitor that corresponds to residues 137 to 157 of c-Src (Sato *et al.*, 1990), lost their ability to undergo the sperm-induced calcium transient. Microinjection study has also been conducted in our lab with the use of peptide A7, a peptide A derivative (Fukami *et al.*, 1993; Sato *et al.*, 1999). In this study, it was shown that not only egg activation events but also penetration of sperm is blocked in the peptide-injected eggs, suggesting that a very early step of fertilization, which might be related to sperm–egg fusion and incorporation of sperm nuclei, is blocked. This is in part consistent with our present result that even one SEP was rarely visible in the PP1-injected eggs (Table 2). In the case of PP1, however, a subset of the inhibitor-injected eggs did show at least one SEP. It surely indicates that in such group of eggs, PP1 allows sperm to fertilize the egg but prevents the subsequent intracellular signaling event. On the other hand, the reason why even one SEP was hardly detectable in another subset of PP1-injected eggs is unknown. One possible explanation for this is that PP1-sensitive kinase is required for incorporation of sperm or the local pigment change upon sperm entry (Palecek *et al.*, 1978). Alternatively, PP1 may cause membrane depolarization and/or an exocytosis of cortical granule in eggs which in turn prevents the successful sperm adhesion/fusion. Further detailed analysis should be done to resolve this problem.

Another important aspect of the PP1 data is the lack of the inhibitory effect on ionophore-induced egg activation events (Table 2). A similar result has also been obtained in our previous study with peptide A7 that also failed to block the A23187-induced cortical contraction of the egg (Sato *et al.*, 1999). These observations suggest that requirement of PTK activity for egg activation can be skipped when $[Ca^{2+}]_i$ is artificially elevated. However, it should also be noted that A23187 does not utilize the IP_3-sensitive calcium stores in initiating calcium signaling as in the case of fertilization. So, it will be important to test whether IP_3-induced calcium release is also able to skip the requirement of egg PTK activity in initiating calcium transient.

Several other PTK inhibitors also have been used to define the requirement of egg PTK activity for the onset of sperm-induced egg activation in marine invertebrates. In sea urchin, genistein causes a delay in sperm-induced calcium transient that would result in polyspermy, suggesting that fertilization-dependent PTK activity may play a role upstream of calcium-dependent events (Moore and Kinsey, 1995; Shen *et al.*, 1999). On the other hand, such defect in the early phase of egg activation was not observed with ascidian eggs treated with genistein (Ueki and Yokosawa, 1997). In this organism, erbstatin was shown to block the cortical reaction in response to fertilization. In mouse egg, it has been reported that genistein is effective in blocking calcium oscillation/spiking in response to fertilization (Dupont *et al.*, 1996). These data, however, do not identify which PTK is working in fertilization. An alternative approach to inhibit specifically the function of egg Src family PTK has been made by the group led by Foltz who found that the Src or Fyn SH2 domain could inhibit the onset of the calcium transient in response to fertilization in sea urchin eggs (Abassi *et al.*, 2000) and starfish eggs (Giusti *et al.*, 1999b). The same group has also demonstrated that Src-related PTK of 57 kDa in sea urchin eggs and of 58 kDa in starfish eggs may play an important role in PLCγ-dependent calcium transient upon fertilization (Abassi *et al.*, 2000; Giusti *et al.*, 1999a). Thus, it is of special interest whether these Src-related PTKs in marine invertebrates are structurally and functionally homologous to Xyk.

We used U-73122 to test if PLC activity is necessary for sperm-induced egg activation processes and the results obtained so far indicate that *Xenopus* egg fertilization involves PLC activity which may act upstream of the calcium-signaling pathway (Fig. 7). To our knowledge, this is the first report showing that PLC activity is necessary for sperm-induced activation of the *Xenopus* egg. As seen for PP1, U-73122 also did not affect the ionophore-induced egg activation processes (Table 2), suggesting that PLC dependency can be overcome in eggs with elevated $[Ca^{2+}]_i$.

The effect of U-73122 on fertilization/egg activation has been described in sea urchin (Lee and Shen, 1998) and mouse (Deng *et al.*, 1998; Dupont *et al.*, 1996). In sea urchin, 10 μM U-73122 caused inhibition of the calcium transient and subsequent fertilization envelope formation. The resulting gametes showed a polyspermic phenotype with an average value of about two sperm nuclei per gamete (Lee and Shen, 1998). This is consistent with our result that microinjection of U-73122 at 10 μM resulted in the appearance of multiple sperm entry points (Table 2). Dupont *et al.* (1996) have reported that in mouse eggs, calcium spiking/oscillation in response to fertilization could be blocked by U-73122 in a dose-dependent manner. In contrast, Deng *et al.* (1998) have reported a result showing the inability of U-73122 to prevent fertilization-induced calcium response in mouse eggs. This controversy may be due to difference of the timing of drug application. In any case, however, it has been unclear which isozyme of PLC is affected in U-73122-treated eggs, because U-73122 has a broad specificity toward all PLC isozymes. In the case of *Xenopus*, sperm-induced IP_3 increase and the calcium transient are prevented in PP1-injected eggs, and therefore, PLCγ seems

to be the most likely target of U-73122. It is, however, still impossible to rule out that other isozymes of PLC (β and δ) are involved in PP1- and U-73122-sensitive PLC activity in fertilized *Xenopus* eggs (Fig. 7). This is due to our inability to determine the active state of PLCβ and δ in the course of fertilization. PLCβ is of special interest, because a recent report has demonstrated that $G_{q/11}$, a well-known activator of PLCβ, can be activated by tyrosine phosphorylation of the α subunit (Umemori *et al.*, 1997), although Runft *et al.* (1999) have argued against a role for G_q in *Xenopus* fertilization.

Recently, some laboratories have examined the isozyme-specific function of PLCs in fertilization. In both sea urchin (Carroll *et al.*, 1999; Shearer *et al.*, 1999) and starfish eggs (Carroll *et al.*, 1997), the SH2 domains of PLCγ have been shown to inhibit the sperm-induced calcium transient when microinjected into eggs. This is in contrast to the results obtained with mouse eggs (Mehlman *et al.*, 1998). Interestingly, in mouse eggs, the SH3 domain of PLCγ was shown to inhibit Tr-Kit-induced parthenogenetic egg activation (Sette *et al.*, 1998), but not the fertilization-induced calcium transient (Mehlmann *et al.*, 1998). The SH2 domains of PLCγ have also been shown to be ineffective in *Xenopus* egg fertilization (Runft *et al.*, 1999), indicating that SH2 domain-mediated activation of PLCγ is not required for calcium release at fertilization of *Xenopus*. So, if tyrosine phosphorylation of PLCγ is in fact required for its enzyme activation and egg activation in *Xenopus* (this study), it would be an SH2-independent event which would involve another type of protein–protein interaction and/or tyrosine phosphorylation of PLCγ itself as demonstrated in this study.

Mechanism of Fertilization-Induced Up-regulation of Xenopus Egg PLCγ

As noted in the Introduction, it is well established that certain kinds of extracellular stimuli such as growth factors (PDGF, EGF, FGF, etc.) and cytokines (interleukins etc.) or ligation of certain cell surface receptors (T cell antigen receptor, integrins, etc.) can stimulate tyrosine phosphorylation and activation of PLCγ within the cells. In mammalian cells, phosphorylation of PLCγ occurs on tyrosine residues 771, 783, and/or 1254 (in mammalian PLCγ) and substitution of these tyrosine residues to phenylalanine blocks the activation of PLCγ (Rhee and Bae, 1997; Singer *et al.*, 1997).

Upon fertilization, tyrosine phosphorylation and activation of PLCγ occurred simultaneously (Fig. 1), and concomitant association of Xyk and PLCγ was also observed (Fig. 2), suggesting that fertilization-induced activation of PLCγ is achieved by its tyrosine phosphorylation catalyzed by Xyk. This scheme is not so surprising, because direct association of Src with PLCγ and activation of PLCγ by Src-mediated tyrosine phosphorylation have been documented in mammalian cells (Nakanishi *et al.*, 1993) and a yeast reconstitution system (Arkinstall *et al.*, 1995). In agreement with the Xyk-PLCγ scheme, PP1 could block almost completely the fertilization-induced tyrosine phosphorylation and activation of PLCγ (Figs. 4A and 4B). In addition, we observed that calcium ionophore-induced egg activation is not accompanied by tyrosine phosphorylation and activation of PLCγ (Fig. 1). This is consistent with our previous finding (Sato *et al.*, 1999) that Xyk is not activated in response to calcium ionophore-induced egg activation.

As to the structural requirement of Src-mediated PLCγ activation, it has been reported that antibodies to the c-Src SH3 domain disrupt the c-Src-PLCγ association and PLCγ activation in the mammalian muscle cells (Marrero *et al.*, 1995) and platelets (Dhar and Shukla, 1994). Our future studies will be directed to define which part of PLCγ molecule is involved in the complex formation with Xyk and whether Xyk can directly phosphorylate and activate PLCγ.

First Signal of Sperm-Induced Activation of Xenopus Eggs

Our present study has been designed to ask if *Xenopus* egg fertilization involves the PTK-PLC pathway and some new insights have been obtained as described above. Nonetheless, we are still far from understanding the mechanism by which fertilizing sperm initiates egg activation processes. This is due to lack of our knowledge about the molecular basis of "first signal" in *Xenopus* egg fertilization (Jaffe, 1996; Whitaker, 1996, 1997). It should be noted that in sea urchin, ascidian, and mouse, soluble sperm factor has been shown to possess egg-activating property which may involve up-regulation of egg PLC activity (Dale, 1988; Parrington *et al.*, 1996, 1999; Sette *et al.*, 1998). On the other hand, in *Xenopus*, it has been suggested that egg components are sufficient for fertilized eggs to undergo egg activation processes. Several recent reports support this with findings that external application of synthetic peptides or protease, which would mimic ligand-related action of sperm, can activate *Xenopus* egg (Iwao and Fujimura, 1996; Mizote *et al.*, 1999; Shilling *et al.*, 1998). In this connection, we have previously reported that a synthetic RGD-containing peptide, a possible egg integrin-interacting peptide, causes egg activation accompanied by activation of Xyk (Sato *et al.*, 1999). The RGD peptide-induced egg activation was shown to be inhibited by PTK inhibitor (Sato *et al.*, 1999). The results imply that stimulation of unknown egg surface receptor (integrin?) can cause egg activation in an endogenous PTK-dependent manner (Fig. 7). So it will be helpful to test whether egg PLCγ could also be under the control of parthenogenetic egg activation that involves egg PTK activity. Further detailed study is necessary to define what kind of cellular components constitute the egg activation machinery of *Xenopus* in response to fertilization.

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REFERENCES

Abassi, Y. A., Carroll, D. J., Giusti, A. F., Belton, R. J., Jr., and Foltz, K. R. (2000). Evidence that Src-type tyrosine kinase activity is necessary for initiation of calcium release at fertilization in sea urchin eggs. *Dev. Biol.* **218,** 206–219.

Abram, C. L., and Courtneidge, S. A. (2000). Src family tyrosine kinases and growth factor signaling. *Exp. Cell Res.* **254,** 1–13.

Arkinstall, S., Payton, M., and Maundrell, K. (1995). Activation of phospholipase Cγ in *Schizosaccharomyces pombe* by coexpression of receptor or nonreceptor tyrosine kinases. *Mol. Cell. Biol.* **15,** 1431–1438.

Brown, M. T., and Cooper, J. A. (1996). Regulation, substrates and functions of src. *Biochim. Biophys. Acta* **1287,** 121–149.

Carroll, D. J., Albay, D. T., Terasaki, M., Jaffe, L. A., and Foltz, K. R. (1999). Identification of PLCγ-dependent and -independent events during fertilization of sea urchin eggs. *Dev. Biol.* **206,** 232–247.

Carroll, D. J., Ramarao, C. S., Mehlmann, L. M., Roche, S., Terasaki, M., and Jaffe, L. A. (1997). Calcium release at fertilization in starfish eggs is mediated by phospholipase Cγ. *J. Cell Biol.* **138,** 1303–1311.

Dale, B. (1988). Primary and secondary messengers in the activation of ascidian eggs. *Exp. Cell Res.* **177,** 205–211.

Deng, M. Q., Huang, X. Y., Tang, T. S., and Sun, F. Z. (1998). Spontaneous and fertilization-induced Ca^{2+} oscillations in mouse immature germinal vesicle-stage oocytes. *Biol. Reprod.* **58,** 807–813.

Dhar, A., and Shukla, S. D. (1994). Electrotransjection of $pp60^{v\text{-}src}$ monoclonal antibody inhibits activation of phospholipase C in platelets. A new mechanism for platelet-activating factor responses. *J. Biol. Chem.* **269,** 9123–9127.

Dupont, G., McGuinness, O. M., Johnson, M. H., Berridge, M. J., and Borgese, 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.

Filtz, T. M., Paterson, A., and Harden, T. K. (1996). Purification and G protein subunit regulation of a phospholipase Cβ from *Xenopus laevis* oocytes. *J. Biol. Chem.* **271,** 31121–31126.

Foltz, K. R., and Shilling, F. M. (1993). Receptor-mediated signal transduction and egg activation. *Zygote* **1,** 276–279.

Fukami, Y., Sato, K.-I., Ikeda, K., Kamisango, K., Koizumi, K., and Matsuno, T. (1993). Evidence for autoinhibitory regulation of the c-*src* gene product: A possible interaction between the Src homology 2 domain and autophosphorylation site. *J. Biol. Chem.* **268,** 1132–1140.

Ghiso, J. A., Alonso, D. F., Farias, E. F., Gomez, D. E., and de Kier Joffe, E. B. (1999). Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur. J. Biochem.* **263,** 295–304.

Giusti, A. F., Carroll, D. J., Abassi,Y. A., and Foltz, K. R. (1999a). Evidence that a starfish egg Src family tyrosine kinase associates with PLC-γ1 SH2 domains at fertilization. *Dev. Biol.* **208,** 189–199.

Giusti, A. F., Carroll, D. J., Abassi, Y. A., Terasaki, M., Foltz, K. R., and Jaffe, L. A. (1999b). Requirement of a src family kinase for initiating calcium release at fertilization in starfish eggs. *J. Biol. Chem.* **274,** 29318–29322.

Glahn, D., Mark, S. D., Behr, R. K., and Nuccitelli, R. (1999). Tyrosine kinase inhibitors block sperm-induced egg activation in *Xenopus laevis*. *Dev. Biol.* **205,** 171–180.

Grey, R. D., Bastiani, M. J., Webb, D. J., and Schertel, E. R. (1982). An electrical block is required to prevent polyspermy in eggs fertilized by natural mating of *Xenopus laevis*. *Dev. Biol.* **89,** 475–484.

Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem.* **271,** 695–701.

Hunter, T. (1998). The phosphorylation of proteins on tyrosine: Its role in cell growth and disease. *Philos. Trans. R. Soc. London B Biol. Sci.* **353,** 583–605.

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.

Jaffe, L. A. (1996). Egg membranes during fertilization. In "Molecular Biology of Membrane Transport Disorders" (S. G. Schultz, T. E. Andreoli, A. M. Brown, D. M. Fambrough, J. F. Hoffman, and M. J. Welsh, Eds.), pp. 367–378. Plenum, New York.

Kanner, S. B., Grosmaire, L. S., Ledbetter, J. A., and Damle, N. K. (1993). β_2-Integrin LFA-1 signaling through phospholipase C-γ1 activation. *Proc. Natl. Acad. Sci. USA* **90,** 7099–7103.

Kline, D. (1988). Calcium-dependent events at fertilization of the frog egg: Injection of a calcium buffer blocks ion channel opening, exocytosis, and formation of pronuclei. *Dev. Biol.* **126,** 346–361.

Kline, D., Kopf, G. S., Muncy, L. F., and Jaffe, L. A. (1991). Evidence for the involvement of a pertussis toxin-insensitive G-protein in egg activation of the frog, *Xenopus laevis*. *Dev. Biol.* **143,** 218–229.

Kline, D., Simoncini, L., Mandel, G., Maue, R. A., Kado, R. T., and Jaffe, L. A. (1988). Fertilization events induced by neurotransmitters after injection of mRNA in *Xenopus* eggs. *Science* **241,** 464–467.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lee, S.-J., and Shen, S. S. (1998). The calcium transient in sea urchin eggs during fertilization requires the production of inositol 1,4,5-trisphosphate. *Dev. Biol.* **193,** 195–208.

Liu, Y., Bishop, A., Witucki, L., Kraybill, B., Shimizu, E., Tsien, J., Ubersax, J., Blethrow, J., Morgan, D. O., and Shokat, K. M. (1999). Structural basis for selective inhibition of Src family kinases by PP1. *Chem. Biol.* **6,** 671–678.

Longo, F. J. (1997). "Fertilization," 2nd ed. Chapman & Hall, London.

Marrero, M. B., Schieffer, B., Paxton, W. G., Schieffer, E., and Bernstein, K. E. (1995). Electroporation of pp60$^{c\text{-}src}$ antibodies inhibits the angiotensin II activation of phospholipase Cγ1 in rat aortic smooth muscle cells. *J. Biol. Chem.* **270,** 15734–15738.

Mehlmann, L. M., Carpenter, G., Rhee, S. G., and Jaffe, L. A. (1998). SH2 domain-mediated activation of phospholipase Cγ is not required to initiate Ca^{2+} release at fertilization of mouse eggs. *Dev. Biol.* **203,** 221–232.

Mizote, A., Okamoto, S., and Iwao, Y. (1999). Activation of *Xenopus* eggs by proteases: Possible involvement of a sperm protease in fertilization. *Dev. Biol.* **208,** 79–92.

Moore, K. L., and Kinsey, W. H. (1995). Effects of protein tyrosine kinase inhibitors on egg activation and fertilization-dependent protein tyrosine kinase activity. *Dev. Biol.* **168,** 1–10.

Myles, D. G., and Primakoff, P. (1997). Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with the egg. *Biol. Reprod.* **56,** 320–327.

Nakanishi, O., Shibasaki, F., Hidaka, M., Homma, Y., and Takenawa, T. (1993). Phospholipase C-γ_1 associates with viral and cellular *src* kinases. *J. Biol. Chem.* **268,** 10754–10759.

Nuccitelli, R. (1991). How do sperm activate eggs? *Curr. Top. Dev. Biol.* **25,** 1–16.

Nuccitelli, R., Yim, D. L., and Smart, T. (1993). The sperm-induced Ca^{2+} wave following fertilization of the *Xenopus* egg requires the production of Ins(1,4,5)P_3. *Dev. Biol.* **158,** 200–212.

Palecek, J., Ubbels, G. A., and Rzehak, K. (1978). Changes of the external and internal pigment pattern upon fertilization in the egg of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **45,** 203–214.

Parrington, J., Jones, K. T., Lai, A., and Swann, K. (1999). The soluble sperm factor that causes Ca^{2+} release from sea-urchin (*Lytechinus pictus*) egg homogenates also triggers Ca^{2+} oscillations after injection into mouse eggs. *Biochem. J.* **341,** 1–4.

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.

Quill, T. A., and Hedrick, J. L. (1996). The fertilization layer mediated block to polyspermy in *Xenopus laevis:* Isolation of the cortical granule lectin ligand. *Arch. Biochem. Biophys.* **333,** 326–332.

Rhee, S. G., and Bae, Y. S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* **272,** 15045–15048.

Rongish, B. J., Wu, W., and Kinsey, W. H. (1999). Fertilization-induced activation of phospholipase C in the sea urchin egg. *Dev. Biol.* **215,** 147–154.

Runft, L. L., Watras, J., and Jaffe, L. A. (1999). Calcium release at fertilization of *Xenopus* eggs requires Type I IP_3 receptors, but not SH2 domain-mediated activation of PLCγ or Gq-mediated activation of PLCβ. *Dev. Biol.* **214,** 399–411.

Sato, K.-I., Miki, S., Tachibana, H., Hayashi, F., Akiyama, T., and Fukami, Y. (1990). A synthetic peptide corresponding to residues 137 to 157 of p60v-src inhibits tyrosine-specific protein kinases. *Biochem. Biophys. Res. Commun.* **171,** 1152–1159.

Sato, K.-I., Aoto, M., Mori, K., Akasofu, S., Tokmakov, A. A., Sahara, S, and Fukami, Y. (1996). Purification and characterization of a Src-related p57 protein-tyrosine kinase from *Xenopus* oocytes: Isolation of an inactive form of the enzyme and its activation and translocation upon fertilization. *J. Biol. Chem.* **271,** 13250–13257.

Sato, K.-I., Iwasaki, T., Tamaki, I., Aoto, M., Tokmakov, A. A., and Fukami, Y. (1998). Involvement of protein-tyrosine phosphorylation and dephosphorylation in sperm-induced *Xenopus* egg activation. *FEBS Lett.* **424,** 113–118.

Sato, K.-I., Iwao, Y., Fujimura, T., Tamaki, I., Ogawa, K., Iwasaki, T., Tokmakov, A. A., and Fukami, Y. (1999). Evidence for the involvement of a Src-related tyrosine kinase in *Xenopus* egg activation. *Dev. Biol.* **209,** 308–320.

Sato, K.-I., Tokmakov, A. A., and Fukami, Y. (2000). Fertilization signaling and protein-tyrosine kinases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **126,** 129–148.

Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999). Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. *Mol. Cell* **3,** 639–648.

Schmaier, A. H. (1998). Plasma contact activation: A revised hypothesis. *Biol. Res.* **31,** 251–262.

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). Integrins: Emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* **11,** 549–599.

Sekiya, F., Bae, Y. S., and Rhee, S. G. (1999). Regulation of phospholipase C isozymes: Activation of phospholipase C-γ in the absence of tyrosine-phosphorylation. *Chem. Phys. Lipids* **98,** 3–11.

Sette, C., Bevilacqua, A., Geremia, R., and Rossi, P. (1998). Involvement of phospholipase Cγ1 in mouse egg activation induced by a truncated form of the c-kit tyrosine kinase present in spermatozoa. *J. Cell Biol.* **142,** 1063–1074.

Shearer, J., Nadai, C. D., Emily-Fenouil, F., Gache, C., Whitaker, M., and Ciapa, B. (1999). Role of phospholipase Cγ at fertilization and during mitosis in sea urchin eggs and embryos. *Development* **126,** 2273–2284.

Shen, S. S., Kinsey, W. H., and Lee, S.-J. (1999). Protein tyrosine kinase-dependent release of intracellular calcium in the sea urchin egg. *Dev. Growth Differ.* **41,** 345–355.

Shilling, F. M., Magie, C. R., and Nuccitelli, R. (1998). Voltage-dependent activation of frog eggs by a sperm surface disintegrin peptide. *Dev. Biol.* **202,** 113–124.

Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997). Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu. Rev. Biochem.* **66,** 475–509.

Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A., and Bleasdale, J. E. (1990). Receptor-coupled signal transduction in human polymorphonuclear neutrophils: Effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J. Pharmacol. Exp. Ther.* **253,** 688–697.

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

Snow, P., Yim, D. L., Leibow, J. D., Saini, S., and Nuccitelli, R. (1996). Fertilization stimulates an increase in inositol trisphosphate and inositol lipid levels in *Xenopus* eggs. *Dev. Biol.* **180,** 108–118.

Stith, B. J., Epinoza, R., Roberts, D., and Smart, T. (1994). Sperm increases inositol 1,4,5-trisphosphate mass in *Xenopus laevis* eggs preinjected with calcium buffers or heparin. *Dev. Biol.* **165,** 206–215.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211,** 157–176.

Swann, K., and Parrington, J. (1999). Mechanism of Ca^{2+} release at fertilization in mammals. *J. Exp. Zool.* **285,** 267–275.

Thomas, S. M., and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13,** 513–609.

Ueki, K., and Yokosawa, H. (1997). Evidence for an erbstatin-sensitive tyrosine kinase functioning in ascidian egg activation. *Biochem. Biophys. Res. Commun.* **238,** 130–133.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203–212.

Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K., and Yamamoto, T. (1997). Activation of the G protein $G_{q/11}$ through tyrosine phosphorylation of the α subunit. *Science* **276,** 1878–1881.

van der Geer, P., Hunter, T., and Lindberg, R. A. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* **10,** 251–337.

Whitaker, M. (1996). Control of meiotic arrest. *Rev. Reprod.* **1,** 127–135.

Whitaker, M. (1997). Calcium and mitosis. *Prog. Cell Cycle Res.* **3,** 261–269.

Yanagimachi, R. (1994). Mammalian fertilization. In "The Physiology of Reproduction" (E. Knobil and J. D. Neill, Eds.), 2nd ed., pp. 189–317. Raven Press, New York.

Yim, D. L, Opresko, L. K., Wiley, H. S., and Nuccitelli, R. (1994). Highly polarized EGF receptor tyrosine kinase activity initiates egg activation in *Xenopus. Dev. Biol.* **162,** 41–55.

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