Activation of Src and release of intracellular calcium by phosphatidic acid during *Xenopus laevis* fertilization

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ABSTRACT

We report a new step in the fertilization in *Xenopus laevis* which has been found to involve activation of Src tyrosine kinase to stimulate phospholipase C-γ (PLC-γ) which increases inositol 1,4,5-trisphosphate (IP3) to release intracellular calcium ($[Ca]_i$). Molecular species analysis and mass measurements suggested that sperm activate phospholipase D (PLD) to elevate phosphatidic acid (PA). We now report that PA mass increased 2.7 fold by 1 min after insemination and inhibition of PA production by two methods inhibited activation of Src and PLCγ, increased $[Ca]_i$ and other fertilization events. As compared to 14 other lipids, PA specifically bound *Xenopus* Src but not PLCγ. Addition of synthetic PA activated egg Src (an action requiring intact lipid rafts) and PLCγ as well as doubling the amount of PLCγ in rafts. In the absence of elevated $[Ca]_i$, PA addition elevated IP3 mass to levels equivalent to that induced by sperm (but twice that achieved by calcium ionophore). Finally, PA induced $[Ca]_i$ release that was blocked by an IP3 receptor inhibitor. As only PLD1b message was detected, and Western blotting did not detect PLD2, we suggest that sperm activate PLD1b to elevate PA which then binds to and activates Src leading to PLCγ stimulation, IP3 elevation and $[Ca]_i$ release. Due to these and other studies, PA may also play a role in membrane fusion events such as sperm–egg fusion, cortical granule exocytosis, the elevation of phosphatidylinositol 4,5-bisphosphate and the large, late increase in sn 1,2-diacylglycerol in fertilization.

Introduction

In fertilization of *Xenopus laevis,* sperm activate Src which in turn activates phospholipase C-γ (PLCγ) to induce the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI45P2) to inositol 1,4,5-trisphosphate (IP3) and sn 1,2-diacylglycerol (DAG) (Sato et al., 2006). IP3 would then bind to intracellular receptors to cause the release of intracellular calcium ($[Ca]_i$) and activation of fertilization

events (Nader et al., 2013). Src family tyrosine kinases and PLCγ are involved in external fertilization in other species: sea urchin, starfish, ascidian, annelids, and fish (Kinsey, 2013; McGinnis et al., 2011b; Moore and Kinsey, 1994; Satoh and Garbers, 1985; Stricker et al., 2010). We now provide evidence that sperm elevate phosphatidic acid (PA) to activate Src during *Xenopus* fertilization.

A comparison of the mass increases in IP3 (Stith et al., 1992a, 1994, 1993) and DAG (Stith et al., 1992b, 1991) from *Xenopus* oocyte maturation through fertilization, and first mitosis and cleavage, provide insight into lipid signaling during these crucial developmental periods. The IP3 mass increase at fertilization begins by 1 min after insemination, and is larger than those IP3 increases during oocyte maturation, first mitosis or first cleavage. The IP3 increase takes place during the sperm-induced wave of elevated $[Ca]_i$ and cortical granule exocytosis (Stith et al., 1994, 1993). Induction of polyspermy (entry of ~75 sperm) did not increase IP3 mass over that produced after entry of one sperm and this suggests that much of the IP3 increase occurs during the calcium wave, not at the sperm–egg binding site (Stith et al., 1993). Although PLC can be activated by elevated $[Ca]_i$ (Rhee, 2001), sperm stimulate PLC in the absence of elevated $[Ca]_i$ and prevention of the $[Ca]_i$ increase actually results in a larger increase in IP3 mass (suggesting that the $[Ca]_i$ increase stimulates IP3 metabolism) (Stith et al., 1994; Stith, unpublished manuscript). As another

measure of the role of $[Ca]_i$ in PLC activation, calcium ionophore only increases IP3 mass to levels less than half that induced by sperm (Stith et al., 1993).

There is a $[Ca]_i$-independent increase in DAG mass at fertilization and we recorded membrane translocation for two isoforms of protein kinase C (PKC) (Stith et al., 1997). However, the DAG increase (48 pmol) occurred later than that of IP3 and was ~300 times larger (Stith et al., 1997). Due to these disparities, that the DAG increase at fertilization is ~50 times larger than the amount of PI45P2 present (Snow et al., 1996), and since choline mass increased at ~1 min, we suggested that 99+% of the late DAG increase does not originate from PI45P2 hydrolysis by PLC but may be due to phospholipase D (PLD) activation (Stith et al., 1997). PLD catalyzes the degradation of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline, and PA can be dephosphorylated by Lipin to DAG (Bocckino and Exton, 1996; Exton, 1994; Martin et al., 1994; Quest, 1996; Reue and Brindley, 2008).

Our subsequent molecular species analysis supported the role for PA: DAG produced at fertilization has monounsaturated fatty acids (Petcoff et al., 2008) which suggests that DAG originates from PC (DAG derived from PI45P2 is largely made up of highly polyunsaturated fatty acids such as 20:4 and 22:6)(Madani et al., 2001; Wakelam, 1998). Specifically, the increase in DAG is largely due to 18:1n9 DAG and there is a similar decline in the amount of this species in PC (Petcoff et al., 2008). As the monounsaturated form of DAG is believed to be unable to stimulate PKC (Madani et al., 2001), this neutral lipid may have other roles in fertilization such as membrane fusion during cortical granule exocytosis.

In addition to being a source for DAG, PA itself may play a direct or indirect role in membrane fusion between sperm and egg or between the plasma membrane and cortical granules (i.e., exocytosis) (Bearer and Friend, 1982; Chapman, 2008; Choi et al., 2006; Domino et al., 1989; Mendonsa and Engebrecht, 2009; Roth, 2008; Sudhof and Rothman, 2009; van den Bogaart et al., 2012; Vitale et al., 2001; Yang and Frohman, 2012).

We report now that PA mass increased during fertilization and that two PLD inhibitors reduced activation of Src, PLC, $[Ca]_i$ release, gravitational rotation and first cleavage. Gravitational rotation, where the less dense dark pole of the zygote rotates upward, is a measure of cortical granule exocytosis (Kline, 1988). Furthermore, we find that Xenopus Src (but not PLCγ) binds more strongly to PA than 14 other lipids, and addition of synthetic PA activates Src, PLCγ and $[Ca]_i$ release and doubles the amount of PLCγ in low density, detergent resistant membrane rafts (DRM). These data support a model whereby sperm activate PLD1b to produce PA which activates Src to induce fertilization events (Fig. 13).

Materials and methods

Xenopus laevis (Daudin) females were obtained from Xenopus One (Ann Arbor, MI, USA), Xenopus Express (Homosassa, FL, USA) or NASCO (Salida, CA, USA). Sperm (167 μl containing 9.47 million sperm) were added to 1 ml of eggs per published protocols (Petcoff et al., 2008; Stith et al., 1994, 1993, 1997). On the day of use, fresh Modified Barth's Solution (MBS) (pH 7.5; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 0.82 mM MgSO$_4$·7H$_2$O, 0.33 mM Ca(NO$_3$)$_2$·4H$_2$O, 0.41 mM CaCl$_2$·2H$_2$O) was prepared and fertilization was conducted in 10% MBS. Animal protocols have been approved by the University of Colorado Denver IACUC (#86612 (01)1D, expiration date: 1/18/2015). To record the percent of cells undergoing fertilization events, the percentage and time of gravitational rotation (the result of cortical granule exocytosis) and first cleavage was recorded. Under control conditions, we did not see evidence of polyspermy (multiple cleavage furrows).

PA mass measurement

Lipids were extracted with ice cold 1:2 chloroform:methanol (v/v) with BHT, and PA mass was determined with an HPLC (HXPL Rainen, Varian, Santa Clara, CA, USA) and a Sedex 55 evaporative light scattering mass detector (Holland et al., 2003; Stith et al., 2000). PA detector peaks were integrated (Dynamax Method Manager 1.4 software, Rainen), and PA mass calculated with known amounts of standards (Avanti Polar Lipids, Alabaster, AL, USA) and a PA mass of 695 Da.

IP3 assay

PLC activity was recorded by measurement of IP3 mass determined by experimental sample IP3 displacement of labeled IP3 from a calf brain receptor preparation (PerkinElmer Life Sciences, Boston, MA, USA)(Stith et al., 1992a, 1994, 1993). Briefly, IP3 was acid extracted, acid was removed using 600 μl of 3:1 freon:tri-N-octylamine, and the release of [^{3}H]IP3 induced by cold IP3 in the experimental sample was quantified by liquid scintillation. To minimize variation between IP3 receptor preparations and determine the IP3 mass per Xenopus egg, standards were analyzed with each set of experimental samples (Stith et al., 1992a).

Addition of lipids

Groups of Xenopus eggs were incubated with 400 μM 1,2-dicapryloyl-sn-glycero-3-Phosphate (dPA) or control 400 μM 1,2-dioctanoyl-sn-glycero-3-[phospho-L-serine] (dPS) (Avanti Polar Lipids). In some experiments, cells were treated with 250 μM BAPTA-AM (Calbiochem, La Jolla, CA, USA) or 6.1% DMSO (the carrier for BAPTA-AM) for 1 h, then 400 μM dPA (10 min) was added.

Western blotting in whole cells and raft fractions

For determination of the active, tyrosine phosphorylated forms of Src and PLCγ, cells were homogenized in lysis buffer: 5% BME, 3.5 mM SDS, 200 mM PMSF, 2.5 mM EDTA, Protease Arrest KP14001 (Calbiochem, San Diego, CA, USA), 0.3% Phosphatase Inhibitor Cocktail 2 P5726 (Sigma-Aldrich Corp., St. Louis, MO, USA), 88% Phosphosafe Extraction Reagent; 71296-3 (Novagen, Billerica, MA, USA), Freon extracted to remove yolk proteins. Following centrifugation (10 min, 13,991 × g), sample buffer (NP0008, Invitrogen, Life Technologies, Grand Island, NY, USA) was added and samples boiled. After electrophoresis and transfer to 0.45 μm Immobilon-FL PDVF membrane (Millipore, Billerica, MA, USA), antibodies to Src phosphorylated on tyrosine 416/418 (44-660G, Invitrogen) or PLCγ1 phosphorylated on tyrosine 783 (AB4828, Abcam, Boston, MA, USA) were used to detect the activated forms of the enzymes (McGinnis et al., 2011b; Sato et al., 2002, 2001, 2003, 2000, 2010; Tokmakov et al., 2002). After use of Odyssey Blocking Buffer (927-40000, LI-COR Biosciences, Lincoln, NE, USA), goat anti-rabbit polyclonal antibody conjugated to an infrared fluor (at 700 nm: 926-32220, and 926-32211 at 800 nm; LI-COR Biosciences) was added and detection utilized an Odyssey two-laser infrared imaging system (LI-COR). In addition, a phosphoSrc ELISA was used as a second method to record Src activation (KHO 0171, Biosource International/Life Technologies).

A "pan PLD" antibody against all isoforms of PLD (conjugated to KLH; gift from Dr. David Brindley; University of Alberta, Edmonton, Canada) was used. To detect PLD1b, a custom antibody was generated by use of a peptide that spans the splice site present only in the 1b isoform of PLD (SIDSESHRGSVR; see Fig. 12) (Open Biosystems/Thermo Scientific, Waltham, MA, USA). We also used four commercially available polyclonal, affinity purified PLD