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Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate

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ABSTRACT

Acrosomal exocytosis involves a massive fusion between the outer acrosomal and the plasma membranes of the spermatozoon triggered by stimuli that open calcium channels at the plasma membrane. Diacylglycerol has been implicated in the activation of these calcium channels. Here we report that this lipid promotes the efflux of intraacrosomal calcium and triggers exocytosis in permeabilized human sperm, implying that diacylglycerol activates events downstream of the opening of plasma membrane channels. Furthermore, we show that calcium and diacylglycerol converge in a signaling pathway leading to the production of phosphatidylinositol 4,5-bisphosphate (PIP_2). Addition of diacylglycerol promotes the PKC-dependent activation of PLD1. Rescue experiments adding phosphatidic acid or PIP_2 and direct measurement of lipid production suggest that both PKC and PLD1 promote PIP_2 synthesis. Inhibition of different steps of the pathway was reverted by adenophostin, an agonist of IP_3-sensitive calcium channels, indicating that PIP_2 is necessary to keep these channels opened. However, phosphatidic acid, PIP_2, or adenophostin could not trigger exocytosis by themselves, indicating that diacylglycerol must also activate another factor. We found that diacylglycerol and phorbol ester stimulate the accumulation of the GTP-bound form of Rab3A. Together our results indicate that diacylglycerol promotes acrosomal exocytosis by i) maintaining high levels of IP_3 – an effect that depends on a positive feedback loop leading to the production of PIP_2 – and ii) stimulating the activation of Rab3A, which in turn initiates a cascade of protein interactions leading to the assembly of SNARE complexes and membrane fusion.

1. Introduction

Regulated exocytosis is a process by which secretory vesicles are directed to the plasma membrane to subsequently discharge their contents into the extracellular environment upon calcium-triggered opening of fusion pores [1]. Many molecules belonging to or associated with the fusion machinery have been identified and characterized. These proteins include GTPases of the Rab family [2], the sec1p/Munc-18 protein family [3], and integral components of the SNARE complex present in vesicles and the plasma membrane, in addition to NSF and α-SNAP [4]. SNAREs have been recognized as key components of protein complexes that drive membrane fusion. They provide the energy required to fuse membranes through the formation of parallel and high affinity four-α-helix bundles [4]. The exocytotic process is highly regulated through the orchestrated actions of various proteins and lipids [1]. For instance, diacylglycerol (DAG), a phospholipid cleavage product generated by phospholipase C activity, can trigger the fusion of liposomes [5], and is required for the fusion of isolated organelles [6] and mediates vesicle fusion through the activation of a TRP channel [7]. It is well known that phorbol esters, functional analogs of DAG, potently enhance neurotransmitter release at synapses [8]. Further, Xue et al. demonstrated the involvement of PKCα in phorbol 12-myristate 13-acetate (PMA)-induced facilitation of exocytosis and vesicle fusion in PC12 cells [9]. Phorbol esters and DAG activate C1-domain-containing proteins, such as PKCs and Munc13 [10] both known to be important players during membrane fusion. Phosphoinositides are capable to bind fusion proteins in a common microdomain [11] regulating SNARE dependent fusion [12]. Sterols support homotypic vacuole fusion in yeast [13]. The need for different lipids for membrane fusion underscores the role of these molecules in organizing the membrane elements of this complex reaction.

Exocytosis of the sperm's acrosome – also called acrosome reaction – is a synchronized process that happens only once in the life of the cell.

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The opening of multiple fusion pores results in the progressive loss of the outer acrosomal and plasma membrane causing the release of the hydrolytic enzymes stored in the acrosomal granule. We have shown that sperm exocytosis utilizes a conserved exocytotic machinery similar to that characterized in other secretory cells (reviewed in [14]). Acrosomal exocytosis can be triggered by sperm contact with physiological stimuli such as progesterone or the zona pellucida. Upon sperm activation, a complex signal transduction cascade, which includes voltage-operated Ca^{2+} channels (VOCC), store-operated Ca^{2+} channels (SOCCs), and inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} channels leads to a sustained cytosolic Ca^{2+}increase which initiates the acrosomal exocytosis [15–17].

Addition of DAG or membrane-permeant phorbol esters triggers acrosomal exocytosis in spermatozoa of different species [18–21]. It has also been demonstrated that DAG formation during acrosomal exocytosis is differentially regulated by transducing pathways activated by physiological agonists [22]. Therefore, DAG plays a key role in events leading to membrane fusion during sperm acrosomal exocytosis but its molecular mechanism of action has remained unknown. DAG/PMA has been involved in the opening of plasma membrane calcium channels during the signal transduction cascade in acrosomal exocytosis [23,24]. The TRP superfamily of ion channels, which are regulated by DAG, has been demonstrated to be central to the regulation of fertilization [25]. Taking into account DAG multiple actions we decided to explore if this lipid plays additional roles downstream of the opening of plasma membrane calcium channels. We used a streptolysin O (SLO) permeabilization protocol developed in our laboratory for human sperm cells to bypass any effect of this lipid on the regulation of calcium channel operating in this membrane [26–28].

Our results indicate that DAG triggers acrosomal exocytosis by promoting two processes: production of PIP_2 by activating a positive feedback loop involving PKC and PLD1, and the activation of Rab3A. Our observations contribute to broaden the vision about the role of lipids in human sperm acrosomal exocytosis and consequently in fertilization.

2. Materials and methods

2.1. Materials

Streptolysin O (SLO) was obtained from Dr. Bhakdi (University of Mainz, Mainz, Germany). Chelerythrine, U73122, U73343, Ro-31-7549, adenophostin, 2-aminoethoxydiphenylborate (2-APB), xestospongin C, isopropyl β-D-1-thiogalactopyranoside (IPTG), glutathione, FITC conjugated *Pisum sativum* agglutinin (FITC-PSA) and EGTA were from Merck Química Argentina SAIC (Buenos Aires, Argentina); glutathione sepharose 4B was from GE Healthcare, (Buenos Aires, Argentina). Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12-myristate 13-acetate (αPMA), 1,2-dioctanoyl-sn-glycerol (catalog number D5156), phosphatidic acid (PA), FIPI hydrochloride hydrate, and A23187 were from Sigma-Aldrich Argentina SA, (Buenos Aires, Argentina); phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP), were from Avanti Polar Lipids, Inc., (Alabaster, USA); EGTA-AM, Fluo3-AM, and anti-PIP_2 monoclonal antibody 2C11 (catalog number A21327) were from Molecular Probes, Invitrogen Corporation, (Buenos Aires, Argentina); [γ-^{32}P]ATP and Chemiluminescence Reagent Plus were from Perkin Elmer Life And Analytical Sciences, Inc., Migliore Laclaustra SRL (Buenos Aires, Argentina); 1-butanol was from BDH Chemicals; anti-Rab3A was from Synaptic Systems (mouse monoclonal antibody, purified IgG clone 42.2); anti-PLD1 (polyclonal rabbit anti-human PLD1, ab10585) and anti-Syt VI (rabbit anti-human Synaptotagmin VI) were from Abcam plc (Cambridge, UK), anti-SNAP25 was from Synaptic Systems (monoclonal mouse antibody, clone 71.1; conserved epitope), Cy-3-conjugated anti-rabbit and HRP goat-conjugated anti-rabbit were from Jackson IR, Inc., USA. CAY10593 (N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methylethyl]-2-naphthalenecarboxamide) was from Cayman Chemical (Michigan, USA). The plasmid pEGFP-PLCδ$_1$-PH, which was later subcloned in a pQE80L was a kind gift from Dr. Tobias Meyer (Stanford University School of Medicine, Stanford, CA, USA) and pGEX-4T-1-PABD was described in Kassas et al. [29]. Purified R-Rab3A, botulinum neurotoxin E (BoNT/E), and GST-RIM were provided by Matías Bustos from IHEM-CONICET—Universidad Nacional de Cuyo, Mendoza, Argentina.

2.2. Protein purification

The plasmids pQE80L-PLCδ1-PH and pGEX-4T-1-PABD were transformed into *Escherichia coli* strain BL21(DE3)pLysS. Protein synthesis was induced overnight at 22 °C with 0.5 mM IPTG. Bacteria were centrifuged and lysed by sonication and proteins were purified under native conditions on glutathione-Sepharose 4B and eluted with 20 mM glutathione in 0.2 M NaCl, 50 mM Tris–HCl pH 8.

2.3. Acrosome reaction assay

Human semen samples were obtained from healthy donors. Highly motile sperm were recovered after a swim-up separation for 1 h in HTF (5.94 g/l NaCl, 0.35 g/l KCl, 0.05 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l KH_2PO_4, 0.3 g/l $CaCl_2 \cdot 2H_2O$, 2.1 g/l $NaHCO_3$, 0.51 g/l glucose, 0.036 g/l Na pyruvate, 2.39 g/l Na lactate, 0.06 g/l penicillin, 0.05 g/l streptomycin, 0.01 g/l phenol red supplemented with 5 mg/ml of bovine serum albumin) at 37 °C in an atmosphere of 5% CO_2/95% air. Cell concentration was then adjusted with HTF to 5–10 × 10^6 sperm/ml. To promote capacitation of the motile fraction recovered from the swim-up procedure, sperm were incubated in HTF supplemented with 5 mg/ml of bovine serum albumin for at least 3 h at 37 °C in an atmosphere of 5% CO_2/95% air. Capacitated spermatozoa were used without any further treatment (pull down assays) or permeabilized. Permeabilization was performed with 2.1 units/ml SLO in HB-EGTA (20 mM HEPES-K, 250 mM sucrose, 0.5 mM EGTA, pH 7). Inhibitors or stimulants were added as indicated in the figure legends. Samples for each condition were air-dried, fixed/permeabilized with −20 °C methanol, and stained with 50 μg/ml FITC-PSA in PBS for 40 min at room temperature [30]. Then the cells were washed with distilled water for 20 min at 4 °C. At least 300 cells were scored using a Nikon microscope equipped with epifluorescence optics. Negative (no stimulation) and positive (stimulated with 0.5 mM $CaCl_2$ corresponding to 10 μM free calcium estimated by MAXCHELATOR) controls were included in all experiments. For each experiment, acrosomal exocytosis indexes were calculated by subtracting the number of reacted spermatozoa in the negative control (range 6.6–22%) from all values and expressing the resulting values as a percentage of the acrosome reaction observed in the positive control (range 15–45%). The average difference between positive and negative control was 14% (experiments where the difference was <10% were discarded).

2.4. Lipids added to spermatozoa

2.4.1. Diacylglycerol

100 mM DAG in DMSO stock solution was kept at −20 °C until used. Successive dilutions in HB-EGTA were done to reach a final DAG concentration of 0.5 mM. One microliter of the 0.5 mM solution was added to 49 μl of sperm suspension in HB-EGTA to get a final concentration of 10 μM. The final DMSO concentration was 0.01%.

2.4.2. Phorbol ester

1 M PMA stock in DMSO was prepared and a N_2 stream was applied before storing at −20 °C. We added 0.5 μl of the stock to 49.5 μl of DMSO to obtain a 0.01 M solution. From this stock we prepared additional dilutions in HB-EGTA until getting a 10 μM PMA solution. One microliter of the 10 μM stock was added to 49 μl of sperm suspension to get a final concentration of 200 nM.

2.4.3. Phosphatidic acid

5 mM PA in DMSO stock solution was kept at −20 °C until used. A dilution in HB-EGTA was done to reach a concentration of 0.5 mM. One microliter of the 0.5 mM solution was added to 49 μl of sperm suspension in HB-EGTA to get a final concentration of 10 μM PA. The final DMSO concentration was 0.2%.

2.4.4. Phosphatidylinositol-4,5-bisphosphate

Phosphatidylinositol-4,5-bisphosphate was dissolved in chloroform: methanol:water (20:9:1, v/v), vortexed and evaporated. The lipid was then dissolved in chloroform and dried under a nitrogen stream. PIP_2 is a polar phospholipid with a variable net charge (subject to pH and membrane interactions [31]), that forms micelles in aqueous solution. PIP_2 micelles were made by suspending the lipid in HB-EGTA at a final concentration of 2.5 mM (stock solution), followed by several minutes of sonication at maximum power. One microliter of the 2.5 mM solution was added to 49 μl of sperm suspension in HB-EGTA to get a final concentration of 50 μM PIP_2.

2.5. Imaging of intracellular calcium stores

SLO-permeabilized sperm were incubated for 30 min at 37 °C in the presence of 2 μM Fluo3-AM. The cells were then washed with HB-EGTA. Afterwards, cells were immobilized on poly-L-lysine-coated round coverslips (0.01% w/v poly-L-lysine drops were air dried followed by one rinse with water) which were mounted on a chamber and placed on the stage of an inverted Eclipse TE300 Nikon microscope. LED output was synchronized to the Exposure Out signal of a Luca R EMCCD camera (Andor Technology, UK). Images were collected (3 frames/s) using NIS Element software (Nikon, NY, USA) and a Plan Apo 60×/1.40 -oil- Nikon objective. Images were processed using Image J (National Institutes of Health, http://rsb.info.nih.gov/ij/). Any incompletely adhered sperm that moved during the course of the experiment were discarded. Fluorescence measurements in individual sperm were made by manually drawing a region of interest around the acrosome and midpiece of each cell. Results are presented as pseudo color $[Ca^{2+}]$i images as indicated on the figures. When required, raw intensity values were imported into Microsoft Excel and normalized using F/Fo, where F is fluorescence intensity at time t and Fo is the mean of F taken during the control period. The total series of F/Fo were then plotted vs. time (s). Relative Fluorescence (%) is the fluorescence normalized to that obtained before the diacylglycerol addition.

2.6. SDS PAGE and immunoblotting

30×10^6 sperm cells/condition were treated with 100 μM 2-APB for 15 min to prevent acrosomal exocytosis and membrane loss. Then spermatozoa were left untreated or stimulated either with 10 μM A23187 or 200 nM PMA for another 15 min. After treatment, cells were incubated in hyposmotic buffer (7.35 mg/ml sodium citrate·$2H_2O$, 13.5 mg/ml sucrose) for two hours at 37 °C and sonicated. After sonication cells were centrifuged 15 min at 4000 *g* and 10 min at 10,000 *g* to extract cell debris and nucleus. Postnuclear supernatants were centrifuged 2 h at 100,000 *g*. The pellets were resuspended in a sample buffer. Proteins were separated in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% non-fat dry milk dissolved in washing buffer (0.1% Tween 20 in PBS) for one hour at room temperature. Blots were incubated with 1 μg/ml anti-PLD1 antibody in blocking buffer for 2 h. After washing three times, blots were incubated with 0.25 μg/ml HRP-conjugated anti-rabbit antibody in washing solution for one hour and washed three more times. Equal membrane load was confirmed by immunoblot against synaptotagmin VI. Briefly, blots were stripped with 50 mM HCl at RT and blocked as before. Then blots were incubated for two hours with 2 μg/ml rabbit anti-Synaptotagmin VI in blocking solution. The procedure followed as described above. Detection was accomplished with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). The images of the bands were obtained using a Luminescent Image Analyzer LAS-4000 (Fujifilm).

2.7. Indirect immunofluorescence

3.5×10^5 sperm cells were spotted on polylysine-coated coverslips and fixed with 4% paraformaldehyde in PBS. Free aldehyde groups were quenched with 50 mM glycine in PBS and sperm were permeabilized with 1% Triton X-100 in PBS. Non specific reactivity was blocked with 5% bovine serum. Coverslips were incubated overnight at 4 °C with 10 μg/ml anti-PLD1 antibody diluted in 1% bovine serum in PBS. After 3 washes with PBS, cells were incubated for 1 h at room temperature with 2 μg/ml anti-rabbit Cy-3 conjugated antibody in 1% bovine serum in PBS. Excess of secondary antibody was washed 3 times with PBS. Finally, cells were fixed for 1 min in cold methanol and double stained with FITC-PSA as described in *Acrosome reaction assay* Section 2.3 of Materials and methods. After this procedure, slides were mounted in 1% propyl-gallate/50% glycerol in PBS. Sperm were analyzed with an Eclipse TE-300 Nikon microscope or Nikon Eclipse TE-2000 equipped with a Plan Apo 63/1.40 oil objective and a Hamamatsu (Bridgewater, NJ, USA) Orca 100 camera operated with MetaMorph 6.1 software (Universal Imaging, Downingtown, PA, USA). To detect SNAP25 we followed a similar protocol. Briefly, fixed and permeabilized sperm were incubated overnight at 4 °C with 0.02 μg/μl anti-SNAP25 antibody diluted in 1% BSA in PBS. After 3 washes with PBS, cells were incubated for 1 h at room temperature with 2 μg/ml anti-mouse Cy-3-conjugated antibody in 1% BSA in PBS.

2.8. Thin layer chromatography (TLC)

5×10^7 cells/condition were permeabilized with SLO and treated with 1 mM $MgCl_2$ to stimulate kinase activity, 5 mM NaF to avoid phosphatase activity and 15 μM U73122 to prevent PIP_2 hydrolysis. 0.375 μCi [γ-^{32}P]ATP was added to each aliquot and cells were kept under control conditions or stimulated with 10 μM calcium, 200 nM PMA, or 10 μM PA. Lipids were neutralized with one volume of 1 M HCl and extracted with two volumes of methanol:chloroform (1:1). Phosphoinositides were then resolved on TLC plates with chloroform: acetone:methanol:acetic acid:H_2O (80:30:26:24:10) and visualized by autoradiography.

2.9. PLD activity measurements

Capacitated sperm cells were adjusted to 10,000 cells/condition in HTF medium supplemented with 0.5% albumin and incubated with 100 μM 2-APB for 15 min at 37 °C (to prevent exocytosis). Cells were further incubated with 10 μM A23187, 10 μM DAG or 200 nM PMA for 15 min at 37 °C. Basal PLD activity was measured without the addition of A23187, DAG or PMA. The medium was replaced by 100 μl of ice-cold Tris 50 mM, pH 8.0, and the cells were lysed by sonication. Samples were collected and mixed with an equal amount of Amplex Red reaction buffer (Amplex Red Phospholipase D assay kit, Molecular Probes), and the PLD activity was estimated after 1 h incubation at 37 °C with a Mithras fluorometer (Berthold). A standard curve was established with purified PLD from Streptomyces chromofuscus (Sigma).

2.10. Rab3A-GTP pull down assay

Capacitated sperm (30×10^6 cells) were treated with 100 μM 2-APB. After that, spermatozoa were incubated with 100 nM R-Rab3A (permeant Rab3A [32] for 15 min at 37 °C followed by the addition of 10 μM A23187, 10 μM DAG, 200 nM PMA, 10 μM PA or under control condition (without any stimulus). After 15 min incubation at 37 °C, cells were lysed by sonication at 0 °C in GST pull down buffer (200 mM

NaCl, 2.5 mM $MgCl_2$, 1% (v/v) Triton X-100, 10% glycerol, 1× protease inhibitor mixture (P2714, Sigma), and 50 mM Tris–HCl, pH 7.4). The sonication was repeated twice for 15 s. These whole cell detergent extracts were clarified by centrifugation at 12,000×*g* for 5 min and used immediately. Glutathione-Sepharose beads were washed twice with GST pull down buffer and incubated with bacterial lysates containing GST-RIM-RBD for 1 h at 4 °C under constant rocking. Beads were washed twice with PBS and once with GST pull down buffer and used immediately. 20 μl of glutathione-Sepharose containing 10 μg of the GST-RIM-RBD was added to sperm lysates prepared as described above and incubated by rotation at 4 °C for 45 min. The resin was recovered by centrifugation at 4 °C and washed three times with ice-cold GST pull down buffer. The resin-bound fractions were resolved by SDS-PAGE, and GTP-Rab3A levels were analyzed by immunoblotting. For this purpose we used the following antibodies: mouse monoclonal anti-Rab3A (0.2 μg/ml) and HRP-conjugated goat anti-mouse antibody (1:2000).

2.11. Statistical analysis

Differences between conditions were tested by one-way ANOVA and post-hoc tests like Tukey–Kramer's or Dunnett's. When specified in legends to figures, a Student's *t* test for single group was used.

3. Results

3.1. PMA/DAG is involved in steps occurring downstream of the opening of plasma membrane calcium channels during human sperm acrosomal exocytosis

DAG/PMA has been implicated in the opening of plasma membrane calcium channels during early steps in the signal transduction cascade during acrosomal exocytosis [23,24]. To explore if this lipid plays additional roles downstream of calcium entry, we turned to SLO-permeabilized human sperm. Calcium can freely diffuse through SLO-generated pores abolishing any regulation by plasma membrane calcium channels. In fact, addition of 10 μM free calcium in the incubation medium induced exocytosis (Fig. 1A). When permeabilized human sperm were incubated with 200 nM PMA or 10 μM DAG they underwent acrosomal exocytosis of a similar magnitude to that triggered by 10 μM Ca^{2+} (Fig. 1A and Supplementary Fig. 1A). The inactive analog of PMA, αPMA, was used as a negative control (Fig. 1A). In the absence of added calcium, the free calcium concentration in the reaction mixture (which contains 0.5 mM EGTA) is on the order of 10^{-7} M [33]. When 5 mM EGTA was added to the system, free calcium concentration drops to less than 10 nM. Even at this very low calcium concentration, sperm underwent exocytosis in response to PMA or DAG (Fig. 1A and Supplementary Fig. 1A) suggesting that the mechanism initiated by PMA/DAG does not require calcium in the incubation mixture. Because some of the reagents used were prepared as stock solutions in DMSO, a control using the vehicle alone was introduced (Fig. 1A). The percentage of DMSO utilized was 0.5% (v/v) which is higher than the concentration used when reagents were added. As shown in Fig. 1A, DMSO does not induce exocytosis by itself.

Previous results from our laboratory indicate that acrosomal exocytosis requires the activation of two parallel processes leading to the assembly of trans SNARE complexes and the release of calcium from intracellular stores [28]. To assess whether PMA/DAG triggers exocytosis by the same mechanism, secretion was tested in the presence of 10 μM EGTA-AM, a membrane-permeant calcium chelator which accumulates in the acrosome in permeabilized sperm (Fig. 1A), or two inhibitors of IP_3-sensitive Ca^{2+} channels (2-APB and xestospongin C, Xc, Fig. 1A and Supplementary Fig. 1A). All these reagents inhibited PMA/DAG-induced exocytosis indicating that the release of calcium from intracellular stores was necessary for secretion.

To evaluate if the mechanism initiated by PMA/DAG requires also functional SNAREs, permeabilized sperm were incubated with the light chains of botulinum neurotoxin E (BoNT/E, which cleaves SNAP25) before exocytic stimulus addition. The treatment of permeabilized sperm with the light chains of BoNT/E completely abolished DAG-induced exocytosis (Fig. 1B). This result indicates that DAG acts by activating the fusion machinery and rules out the possibility that DAG causes acrosome content release by membrane destabilization and/or disruption. As a control, to determine whether the BoNT/E used was active/functional, we incubated sperm with the heat-inactivated toxin (3 min at 100 °C) before adding DAG as an exocytic stimulus. The heat-inactivated toxin failed to inhibit the DAG-induced exocytosis (Fig. 1B, ∅ BoNT/E→DAG). The effect of PMA on sperm SNARE assembly and disassembly dynamics during sperm acrosomal exocytosis was also tested by immunofluorescence. Under control conditions (incubation with BoNT/E in the absence of any stimulus), clear immunolabeling for SNAP25 was observed in the acrosomal region of most cells (Supplementary Fig. 1B, panel II and quantification on the right, Fig. 1C) indicating that this SNARE was protected from toxin cleavage in resting spermatozoa. Then, exocytosis was stimulated with 10 μM DAG in the presence of BoNT/E. Under these conditions, the percentage of spermatozoa with acrosomal SNAP25 labeling decreased significantly (Supplementary Fig. 1B, panel IV and quantification on the right, Fig. 1C). Immunostaining experiments corroborated that DAG, similar to calcium, promotes cis complex disassembly of SNAREs rendering toxin-sensitive SNAP25. Additionally, this experiment confirmed that the BoNT/E used was active and cleaved SNAP25.

According to the two-branch hypothesis, inhibition of SNARE complex assembly should not affect the release of calcium from the acrosome. We thus tested the prediction that DAG activates the efflux of calcium from the acrosome in permeabilized cells treated with BoNT/E to prevent exocytosis. Acrosomes were loaded with the calcium indicator Fluo3-AM, which accumulates in intracellular membrane-bound compartments in permeabilized cells. Calcium changes were measured in single cell experiments. Permeabilized cells showed staining at the acrosome region and midpiece (Fig. 1C, top first three panels on the left); very different from the cytoplasmic distribution observed in non-permeabilized spermatozoa (data not shown). As shown in Fig. 1C, D, E and the movie (Supplementary video 1), acrosomal fluorescence decreased immediately after DAG addition. However, the midpiece staining remained unchanged. The spermatozoon midpiece contains mitochondria and was used as an internal control (Fig. 1C, D, E, and Supplementary video 1). Similar results were obtained blocking exocytosis with BoNT/C Supplementary Fig. 1D, E, F. These observations demonstrate that DAG leads to calcium efflux from the acrosome.

3.2. DAG-induced acrosomal exocytosis requires PLD1 activity and phosphatidic acid downstream PKC activation

Next, we explored the possible mechanism by which DAG promotes the release of calcium from the acrosome. DAG and phorbol esters bind to the C1 domain of PKC activating this enzyme [34]. The presence and involvement of PKC in the mammalian sperm acrosome reaction have been previously highlighted [23,35,36]. To test whether PKC might be the DAG effector in permeabilized sperm we used two selective PKC inhibitors. The competitive inhibitor chelerythrine, which acts on the PKC catalytic domain, and Ro-31-7549, that binds to the ATP binding site of PKC. Both inhibitors completely abrogated DAG and PMA-triggered exocytosis in permeabilized spermatozoa (Fig. 2A and Supplementary Fig. 2A) indicating that PKC activation is required downstream of DAG/PMA.

The hydrolysis of phosphatidylcholine (PC) by PLD to produce phosphatidic acid (PA) is involved in several signal transduction pathways triggered by phorbol esters and DAG [37]. Given that PLD, and particularly PLD1, is involved in membrane fusion during regulated exocytosis [38], we analyzed whether PLD was required for PMA/DAG-

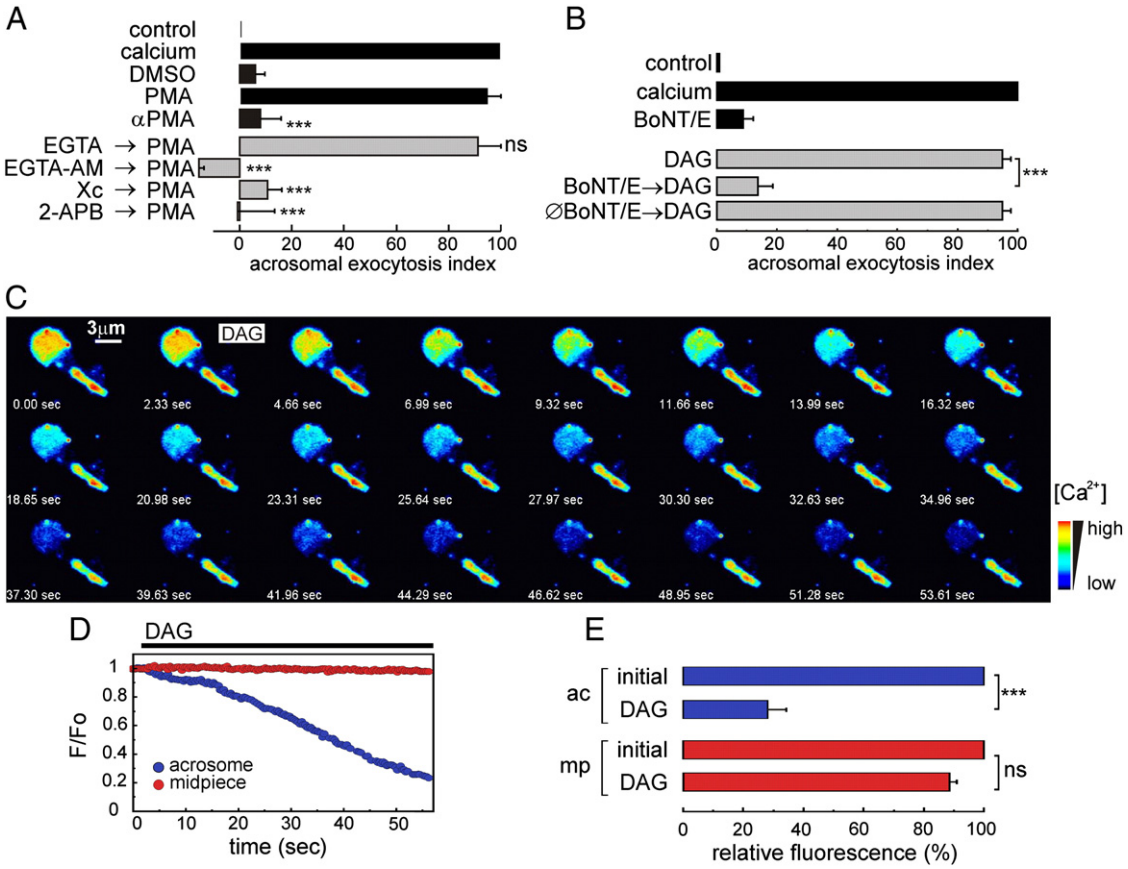

Fig. 1. DAG/PMA is involved in steps downstream of the opening of plasma membrane calcium channels during human sperm acrosomal exocytosis. (A) After capacitation, sperm were permeabilized with SLO and left without any treatment (control) or treated with 10 μM calcium, 0.5% DMSO, 200 nM PMA, or 200 nM αPMA (black bars), and incubated at 37 °C for 15 min. When indicated, cells were treated before the stimulus with 5 mM EGTA, 10 μM EGTA-AM, 1.1 μM xestospongin C (Xc), or 100 μM 2-APB for 15 min at 37 °C. (B) Permeabilized human sperm cells were incubated with or without 300 nM BoNT/E (light chain), to cleave SNAP25, for 15 min at 37 °C. Acrosomal exocytosis was then stimulated with 10 μM DAG for a further 15 min at 37 °C. Several controls were included: background acrosomal exocytosis in the absence of any stimulation (control), heat-inactivated BoNT/E (∅BoNT/E) or BoNT/E treated cells; acrosomal exocytosis stimulated by 10 μM DAG or 0.5 mM $CaCl_2$ (10 μM free calcium, calcium estimated by MAXCHELATOR). Sperm in A and B were fixed and acrosomal exocytosis was evaluated by FITC-PSA binding scoring at least 300 cells per condition. The data – normalized as described in Section 2.3 of Materials and methods – represent the mean ± SEM of at least 5 independent experiments. Dunnett's test was used to compare the means of all groups against the PMA (or DAG) stimulated condition in the absence of inhibitors and classified as non-significant (ns, $P > 0.05$) or significant differences (***, $P < 0.001$). (C, D, and E) Capacitated human sperm recovered after swim-up, permeabilized and incubated with BoNT/E – to avoid acrosomal exocytosis – were incubated for 30 min at 37 °C with 2 μM Fluo3-AM to load intracellular compartments. Cells were then washed and the fluorescence was recorded in an inverted microscope. (C) Representative images of Fluo3-AM-loaded human sperm before (0 and 2.33 s) and after (4.66 to 53.61 s) the treatment with 10 μM DAG. The images are shown in pseudocolor, in which blue and red represent low and high $[Ca^{2+}]i$, respectively. (D) The plot illustrates the traces corresponding to panel C for the acrosomal and midpiece regions. The scales indicate (F/Fo) *vs.* time (s). (E) Relative fluorescence comparing initial values (100%) and 50 s after DAG stimulation in the acrosomal and midpiece regions. Bars represent the mean ± SEM ($N = 15$). Asterisks indicate significant difference (***$P < 0.001$) from the initial value (single group analysis, 99.9% confidence interval).

triggered acrosomal exocytosis. It is well known that the PLD signaling pathway is disrupted in the presence of primary alcohols, since the enzyme catalyzes a transphosphatidylation reaction forming phosphatidylalcohols at the expense of PA [37]. 1-Butanol eliminated the PMA/DAG-triggered acrosome reaction in human spermatozoa (Fig. 2A and Supplementary Fig. 2A), implying the involvement of a PLD in this process. Since secondary and tertiary alcohols are not substrates for PLDs [39], we used the secondary alcohol, isobutanol, as a control (Fig. 2A). As 1-butanol inhibits both PLD isoforms, PLD1 and PLD2, we used CAY10593, which at low concentrations acts as a specific, potent, and selective PLD1 inhibitor [40]. Sperm were incubated with 50 nM CAY10593 before adding PMA (Fig. 2A) or DAG (Supplementary Fig. 2A). Exocytosis was completely abolished in the presence of the PLD1 inhibitor, suggesting that PLD1 is required for acrosomal exocytosis. To confirm this result, we incubated sperm with a polyclonal anti-PLD1 antibody before adding PMA or DAG. The antibody strongly blocked exocytosis, demonstrating the involvement of PLD1 in the acrosome reaction (Fig. 2A, and Supplementary Fig. 2A). The effect was specific since a nonspecific rabbit IgG or the heat-inactivated anti-PLD1 antibody did not affect exocytosis (Supplementary Fig. 2B). Furthermore, if PLD activity is necessary for exocytosis, PA blockers should impair the acrosome reaction. Effectively, incubation with the Phosphatidic Acid Binding Domain (PABD) from the yeast protein Spo20p, which binds tightly to PA, efficiently inhibited the PMA/DAG induced acrosome reaction (Fig. 2A and Supplementary Fig. 2A). Finally, we found that although PA by itself did not trigger exocytosis it efficiently rescued PMA- and DAG-induced exocytosis in the presence of PLD inhibitors (Fig. 2A and Supplementary Fig. 2A), suggesting that PA synthesis is required for the acrosome reaction. Together these results strongly suggest that PA synthesized by PLD1 is necessary for acrosomal exocytosis.

Although in most systems PLD is activated by PKC, in some models PKC has been shown to act downstream of PLD [41]. To unveil if in our system PLD was upstream or downstream of PKC, we assessed whether PA could reverse the effect of the PKC inhibitors chelerythrine and Ro-31-7549 on acrosomal exocytosis. PA overcame the effect of PKC inhibitors, suggesting that PLD activity is necessary after PKC activation in acrosomal exocytosis (Fig. 2A and Supplementary Fig. 2A).

The distribution of PLD1 has been mostly assessed indirectly through overexpression of tagged proteins because of its low abundance, although the endogenous enzyme has been reported in some

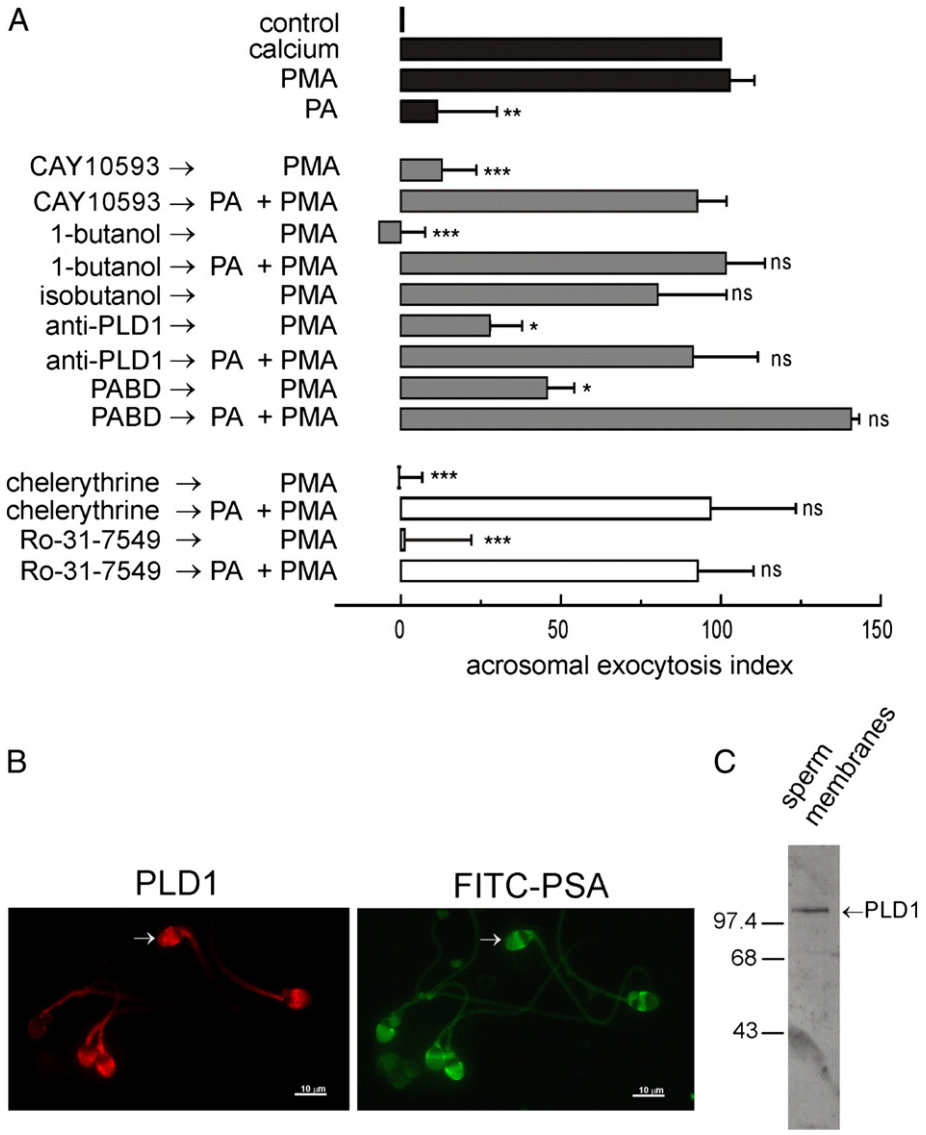

Fig. 2. PMA-induced acrosomal exocytosis requires PLD1 activity and phosphatidic acid downstream of PKC activation. A, human spermatozoa were incubated for 3 h under capacitating conditions and permeabilized as specified in Materials and methods, Section 2.3. The medium was then supplemented, as indicated, with the following compounds affecting phosphatidic acid (PA) availability (gray bars, 50 nM CAY10593, 0.5% 1-butanol, 0.5% isobutanol, 20 μg/ml anti-PLD1 antibody, 10 μg/ml Phosphatidic Acid Binding Domain , PABD) or PKC activity (white bars, 10 μM chelerythrine, 10 μM Ro-31-7549) and incubated for 15 min at 37 °C. The samples were further incubated for 15 min at 37 °C in the presence of 200 nM PMA or PMA plus 10 μM PA. Several controls were run (black bars): permeabilized sperm incubated without any treatment (control) or with 0.5 mM CaCl2 (10 μM free calcium, estimated by MAXCHELATOR), 200 nM PMA, or 10 μM PA. Acrosomal exocytosis was evaluated as explained in Section 2.3. The data represent the mean ± SEM from three to eleven independent experiments. Dunnett's test was used to compare the means of all groups against the PMA stimulated condition in the absence of inhibitors. Significant, (***$P<0.001$; **$P<0.01$; *$P<0.05$) and nonsignificant (ns, $P>0.05$) differences are indicated for each bar. B, indirect immunofluorescence against PLD1 was performed as described in Section 2.7. Briefly, human spermatozoa were fixed and double stained with 10 μg/ml rabbit polyclonal anti-human PLD1 antibody followed by donkey anti-rabbit Cy-3 conjugated antibody (arrow indicates PLD1 staining at the acrosomal region) and FITC-PSA to differentiate between reacted and intact sperm. C, sperm cells were incubated for 3 h under capacitating conditions. Membranes from 30×10^6 cells were obtained according to Bohring and Krause [85]. The samples were analyzed by Western blot using the same anti-human PLD1 antibody used in immunofluorescence. Molecular mass standards (kDa) are indicated on the left.

mammalian cells [42,43]. We searched for the endogenous enzyme in human sperm and found that 80% of the cells displayed specific staining for PLD1 by immunofluorescence; 64% showing an acrosomal pattern (Fig. 2B PLD1, arrow, red staining), 4.5% an equatorial staining, and 58% a midpiece localization. Double-staining with FITC-PSA (Fig. 2B FITC-PSA, arrow, green staining) allowed us to identify the cells with the acrosomal granule intact. Using the same anti-PLD1 antibody we detected a single band of approximately 110 kDa by Western blot (Fig. 2C). These results argue for the expression of PLD1 in human sperm cells and strongly suggest that PLD1 is involved in the acrosomal exocytosis signaling cascade triggered by PMA or DAG after PKC activation.

3.3. PIP_2 is required for PMA/DAG-triggered exocytotic pathway

Recently, Branham et al. [28] have suggested that, in human sperm, cAMP stimulates a cascade initiated by Epac (exchange protein directly activated by cAMP) that activates a PLC, producing IP_3, which then drives the intracellular calcium mobilization required for acrosomal exocytosis. We hypothesized that DAG/PMA might promote the synthesis of PIP_2, providing the substrate for PLC and closing a positive feedback loop that would continuously produce DAG and IP_3. A corollary of this hypothesis is that compounds that sequester PIP_2 should block PMA/DAG-triggered exocytosis. To test this prediction we incubated the cells with the PH domain of PLCδ1 (PH-PLCδ1) [44] or with a specific

anti-PIP_2 antibody before adding PMA or DAG. Results depicted in Fig. 3A show that PMA/DAG-evoked exocytosis was suppressed by >80% in the presence of these proteins (Fig. 3A). The specificity of these reagents was demonstrated by a rescue experiment in which exogenous PIP_2 reversed the effect of these proteins (Fig. 3A). However, PA addition did not reverse the PMA-induced exocytosis abolished by PH-PLCδ1, indicating that PA cannot substitute for PIP_2 (Fig. 3A).

To determine whether the PMA/DAG pathway involves a PLC, we used the PLC thiol-reactive inhibitor U73122 [45]. Fifteen micromolar U73122 inhibited PMA/DAG-induced exocytosis (Fig. 3A), whereas the inactive analog U73343 was ineffective (Fig. 3A). The synthetic lysophospholipid analog; edelfosine, which inhibits phosphatidylinositol PLC, [46] blocked PMA/DAG-induced exocytosis (Fig. 3A) suggesting that a phosphatidylinositol PLC is activated in the DAG-elicited pathway. None of the PLC inhibitors (U73122, U73343, nor edelfosine) showed any effect when added alone (data not shown).

Taken together, these observations suggest that there is a concerted action of PKC and PLD1 that eventually leads to an increase in PIP_2, which is absolutely required for exocytosis. In support of this model we found that PIP_2 reversed the inhibitory effect of 1-butanol, anti-PLD1, chelerythrine, and Ro-31-7549 (Fig. 3B). Furthermore, PIP_2 rescued the inhibition caused by sequestering PA with the PABD domain (Fig. 3B), suggesting that PIP_2 is required for PMA/DAG-elicited exocytosis. These results suggest that PMA/DAG causes an increase in PIP_2 during the signaling cascade leading to the acrosome reaction which is absolutely required for exocytosis. Based on these results, we modeled the effect of DAG on the acrosome reaction as part of a positive feedback mechanism depicted in Fig. 3C.

3.4. Calcium and DAG-induced exocytosis converge at the same signal transduction pathway

The natural trigger for acrosomal exocytosis is a rise in cytoplasmic calcium level. We wondered if calcium could use the pathway characterized for DAG/PMA triggered exocytosis. To test this hypothesis, we first performed functional assays inhibiting PLD and using calcium as the exocytotic stimulus. 1-Butanol as well as anti-PLD1 and PABD abrogated calcium-induced acrosomal exocytosis (Fig. 4A). PA reversed the inhibition of exocytosis caused by 1-butanol, anti-PLD1, and PABD, confirming the specificity of their effect. This reversal stresses the importance of PLD and PA in the calcium-induced exocytotic mechanism (Fig. 4A). The PKC inhibitors chelerythrine and Ro-31-7549 abrogated calcium-triggered exocytosis and PA rescued the process (Fig. 4A), confirming that PKC activity is required for PLD activation and PA production in calcium-induced exocytosis.

To assess whether PIP_2 is required for calcium-induced exocytosis, we incubated the cells with the PH domain of PLCδ1 (PH-PLCδ1) or with a specific anti-PIP_2 antibody before adding calcium. Both proteins inhibited secretion (Fig. 4B) which was rescued by adding PIP_2 (Fig. 4B). If calcium is inducing the synthesis of PIP_2 through the concerted action of PKC and PLD, PIP_2 should be able to reverse the inhibition of these enzymes. Indeed, PIP_2 reversed PLD and PKC inhibition as well as the inhibition caused by sequestering PA with the PABD domain (Fig. 4B). These results indicate that calcium and DAG-induced exocytosis converge on the same signal transduction pathway.

Additionally, we analyzed whether PLD, PKC, and PLC were required for DAG and calcium-induced exocytosis in nonpermeabilized sperm. Both 1-butanol and FIPI, a potent cell-permeable PLD inhibitor, abrogated A23187-induced acrosomal exocytosis in intact cells. Similar results were obtained by blocking PKC (chelerythrine and Ro-31-7549) or PLC (U73122) activity before the A23187 stimulus (Supplemental Fig. 3A). DAG-induced exocytosis in intact spermatozoa also requires the activity of PLD, PKC, and PLC, as shown in Supplementary Fig. 3B. Furthermore, these experiments indicate that permeabilization with SLO was not inducing an artifactual lipid dependence of the acrosomal exocytosis due to decreasing the levels of PA and PIP_2.

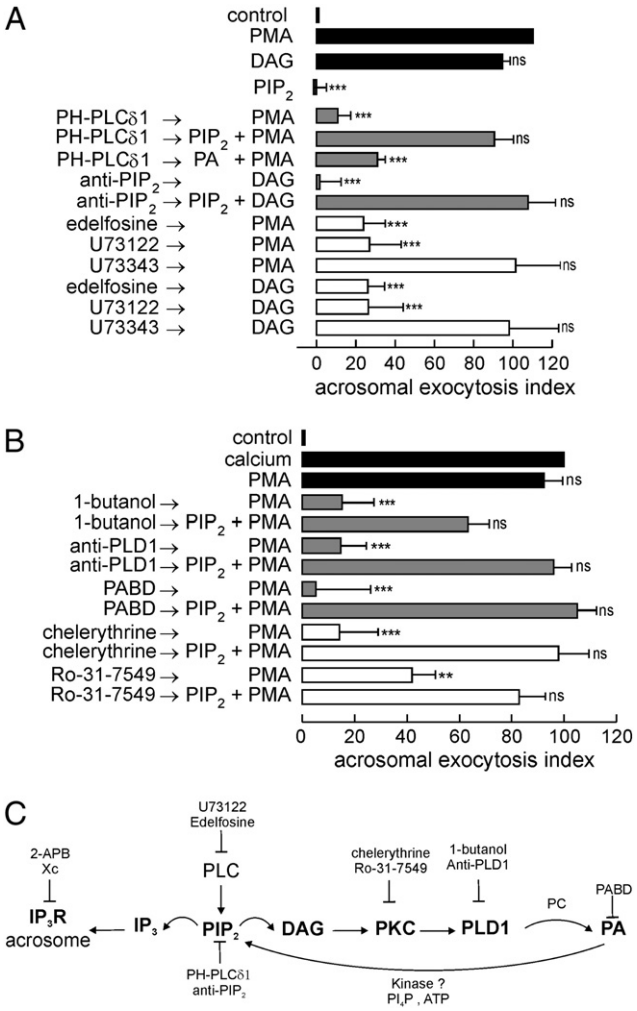

Fig. 3. PIP_2 is required in the PMA/DAG-triggered exocytotic pathway. A, SLO-permeabilized spermatozoa were treated with reagents affecting PIP_2 availability (gray bars, 10 μg/ml PH-PLCδ1 domain, 20 μg/ml antibody anti-PIP_2) or PLC activity (white bars, 0.5 μM edelfosine, 15 μM U73122, 15 μM U73343) for 15 min at 37 °C. When specified, acrosomal exocytosis was activated by adding 200 nM PMA or 10 μM DAG, supplemented with 50 μM PIP_2 or 10 μM PA, and the incubation continued for an additional 15 min at 37 °C. Several controls were performed (black bars): sperm incubated without any treatment (control) or treated with 200 nM PMA, 10 μM DAG, or 50 μM PIP_2. B, SLO-permeabilized spermatozoa were treated with reagents affecting PA availability (gray bars, 0.5% 1-butanol, 20 μg/ml anti-PLD1 antibody, 10 μg/ml Phosphatidic Acid Binding Protein, PABD) or PKC activity (white bars, 10 μM chelerythrine, 10 μM Ro-31-7549) for 15 min at 37 °C. When indicated, the samples were further incubated for 15 min at 37 °C with 200 nM PMA or 200 nM PMA plus 50 μM PIP_2. Several controls were run (black bars): permeabilized sperm incubated without any treatment (control) or stimulated with 10 μM free calcium or 200 nM PMA. For A and B, acrosomal exocytosis was evaluated as explained in Section 2.3. The data represent the mean ± SEM from four to eleven independent experiments. Dunnett's test was used to compare the means of all groups against the PMA (or DAG) stimulated condition in the absence of inhibitors. Significant, $P<0.001$ (***) or nonsignificant $P>0.05$ (ns) differences are indicated for each bar. C, Scheme of the proposed loop. PIP_2 is hydrolyzed after PLC activation generating DAG and IP_3, the latter interacting with IP_3-sensitive calcium channels (IP3R) at the acrosomal membrane. Concomitantly, DAG activates PKC and subsequently PLD1, which breaks down phosphatidylcholine (PC) producing phosphatidic acid (PA) and choline. PA is probably activating a kinase to synthesize PIP_2 from phosphatidylinositol 4-phosphate (PI4P) and ATP closing a positive feedback loop. Additionally, the scheme shows targets for the inhibitors used.

3.5. DAG and calcium increase PLD activity and promote PIP_2 and PIP_3 production

One of the corollaries of the pathway proposed is that calcium and DAG/PMA should activate PLD in human sperm. To assess this proposition we evaluated the activity of the enzyme by measuring the choline released when PLD cleaves phosphatidylcholine under control

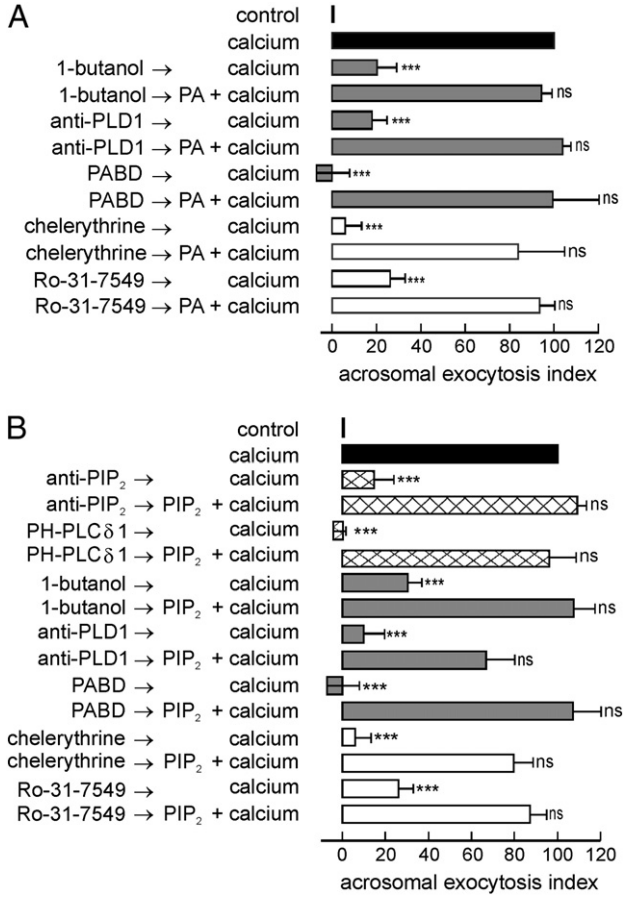

Fig. 4. Calcium and DAG-induced exocytosis converge on the same signal transduction pathway. A, SLO-permeabilized spermatozoa were treated with reagents affecting PA availability (gray bars, 0.5% 1-butanol, 20 μg/ml anti-PLD1 antibody, 10 μg/ml Phosphatidic Acid Binding Protein, PABD) or PKC activity (white bars, 10 μM chelerythrine, 10 μM Ro-31-7549) for 15 min at 37 °C. The samples were further incubated for 15 min at 37 °C with 10 μM calcium supplemented, when indicated, with 10 μM PA. B, SLO-permeabilized spermatozoa were treated with reagents affecting PIP_2 availability (crossed line bars, 20 μg/ml antibody anti-PIP_2, 10 μg/ml PH-PLCδ1 domain), PA availability (gray bars, 0.5% 1-butanol, 20 μg/ml anti-PLD1 antibody, 10 μg/ml Phosphatidic Acid Binding Domain, PABD), or PKC activity (white bars, 10 μM chelerythrine or 10 μM Ro-31-7549) for 15 min at 37 °C. The samples were further incubated for 15 min at 37 °C in the presence of 10 μM calcium supplemented, when indicated, with 50 μM PIP_2. Control conditions are shown in A (black bars, acrosomal exocytosis in the absence of any stimulation or treated with 10 μM calcium). Acrosomal exocytosis was evaluated as explained in Section 2.3. The data represent the mean ± SEM from three to eleven independent experiments. Dunnett's test was used to compare the means of all groups against the calcium stimulated condition in the absence of inhibitors. Significant, $P<0.001$ (***) or nonsignificant $P>0.05$ (ns) differences are indicated for each bar.

conditions and after the incubation of nonpermeabilized sperm cells with DAG, PMA or the calcium ionophore, A23187. As shown in Fig. 5A, PLD activity was significantly increased in spermatozoa stimulated with A23187, DAG, or PMA compared with resting cells. DAG and PMA were found to be more potent activators of PLD than calcium. As shown in Fig. 5A, right, the DAG-induced activity of PLD was significantly inhibited when cells were preincubated in the presence of the PLD specific inhibitor, FIPI.

We wondered whether PLD activation may affect the subcellular localization of the enzyme. Although PLD1 is a protein mostly localized to membranes because of its palmitoyl groups and its pleckstrin and phox homology domains [47], its localization changes when challenged with different compounds. For instance, PLD1 is closely associated with membrane fragments in NRK and GH_3 cells, whereas after inhibition of PA synthesis, PLD1 dissociates from membranes [42]. To analyze whether PLD1 changes its localization with exocytotic stimuli, we obtained total sperm membranes from cells preincubated with PMA or A23187 (Fig. 5B) or not (Fig. 5B, control). Western blot analysis showed a 2–2.4-fold increase of PLD1 in sperm membranes when challenged with PMA or A23187, indicating that both the phorbol ester and calcium are regulating PLD1 distribution between different compartments.

A key prediction of the pathway proposed is that PIP_2 production should increase upon sperm stimulation with calcium or PMA/DAG and that PA is an important mediator of the effect. To evaluate these predictions, permeabilized sperm were incubated with [γ-^{32}P]ATP and the incorporation into phosphoinositides was evaluated by TLC in the presence of the PLC inhibitor U73122, to avoid PIP_2 hydrolysis. Calcium and PMA induced an increase of PIP_2. Notice that PA also provoked a rise in PIP_2 synthesis. Other phosphatidylinositols such as phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) were also synthesized after stimulation (Fig. 5C and D).

Altogether these results strongly validate our hypothesis stating that both calcium and PMA/DAG stimulate the synthesis of PIP_2 through the activation of PLD closing a positive feedback loop that continuously produces DAG and IP_3.

3.6. PIP_2 synthesis stimulated by DAG is required for IP_3-induced acrosomal calcium efflux

We previously demonstrated that an acrosomal calcium efflux after SOCC opening needs to occur for successful exocytosis and cannot be replaced by a cytosolic calcium increase [33,48,49]. In this context, we hypothesized that PIP_2 synthesis is required to maintain a continuous production of IP_3. Although the potent agonist of IP_3 receptors, adenophostin, was not able to induce the acrosome reaction itself, it rescued calcium-induced exocytosis after PKC and PLD inhibition and after sequestering PIP_2 (Fig. 6). Hence it is likely that continued PIP_2 synthesis is necessary to maintain open IP_3-dependent calcium channels in the acrosome, although other functions for the phospholipid cannot be ruled out.

3.7. PMA and DAG stimulate a GTPase exchange factor (GEF) for Rab3A during sperm exocytosis

We have explored the mechanism by which DAG participates in a positive feedback loop that ultimately leads to the release of calcium from the acrosome through IP_3-sensitive calcium channels. However, PA and PIP_2, the phospholipids participating in this mechanism, are not able to trigger exocytosis by themselves. Moreover, the IP_3-sensitive calcium channel agonist adenophostin did not trigger exocytosis (Fig. 6). According to the two-branch model this is not surprising, since acrosome secretion requires the activation of a second cascade involving several proteins, including Rab3A, NSF, SNAREs, complexin, and synaptotagmin. To trigger exocytosis directly, DAG would have to activate this branch in addition to producing IP_3. In Fig. 1B we have shown that DAG-triggered acrosomal exocytosis is sensitive to BoNT/E indicating that this lipid activates the assembly/disassembly dynamics of SNARE complexes that is necessary for exocytosis. The question is then which stage of the process is activated by DAG. Among the various candidates, Rab3A is the earliest factor that has been characterized in the protein branch of sperm exocytosis; moreover, addition of GTP-bound recombinant Rab3A triggers acrosomal exocytosis in permeabilized and intact cells [32,50]. To evaluate if Rab3A is involved in DAG evoked secretion, we introduced anti-Rab3A antibodies in SLO-permeabilized sperm before challenging them with DAG. The antibody inhibited DAG-induced exocytosis indicating that the small GTPase is part of the mechanism stimulated by the lipid (Fig. 7A). If active Rab3A was generated after DAG stimulus, we should be able to inhibit the exocytosis by adding Rab3-GTP binding domain of RIM (amino acids 11–398) [51] to sequester active Rab3A. This domain inhibited the DAG-induced acrosome reaction (Fig. 7A).

DAG has been implicated in the activation of other small GTPases. In fact, the lipid branch of our model includes the small GTPase Rap1

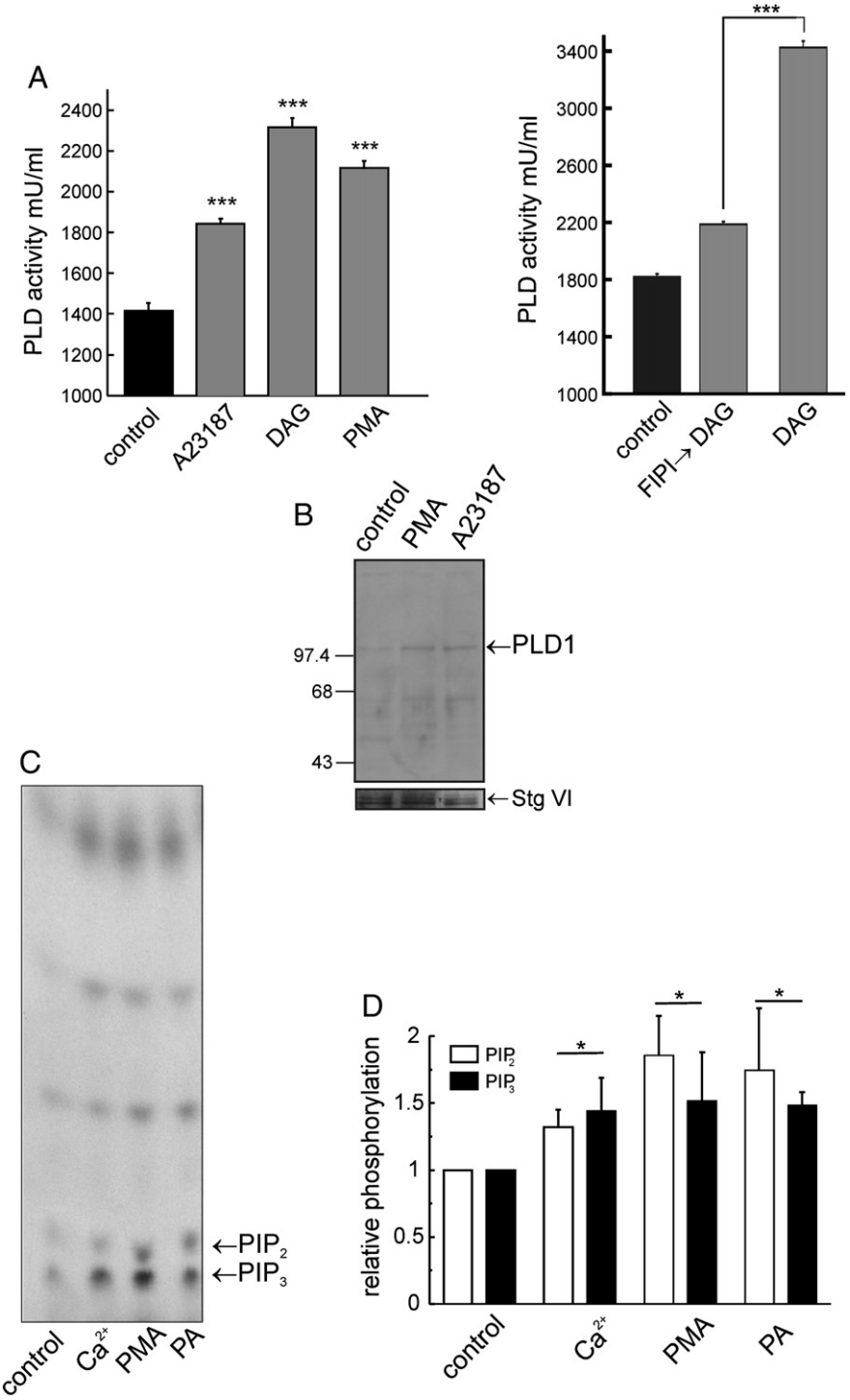

Fig. 5. PMA and calcium increase PLD activity and promote PIP_2 and PIP_3 production in permeabilized spermatozoa. A, for PLD activity measurements, 10,000 cells per condition were incubated in the absence (control, black bar) or presence of 10 μM A23187, 10 μM DAG or 200 nM PMA (gray bars) for 15 min at 37 °C. Spermatozoa were incubated with 100 μM 2-APB before the treatment to avoid acrosomal exocytosis. When indicated, sperm were incubated for 15 min at 37 °C with 7.5 nM FIPI before adding 10 μM DAG (*right*). Cells were collected, lysed and then assayed for PLD activity as described in Section 2.9. Data represent the mean values ± SEM of 4 independent experiments. Dunnett's test was used to compare the means of all groups against the control condition in the absence of any stimulus. Significant differences are indicated for each bar, $P<0.001$ (***). B, 30×10^6 spermatozoa incubated for 3 h under capacitating conditions were treated with 100 μM 2-APB for 15 min and left untreated (control) or treated with 200 nM PMA or 10 μM A23187 for a further 15 min at 37 °C. After that, sperm membranes were extracted according to Bohring and Krause [85]. The samples were analyzed by Western blot using the anti human PLD1 antibody. The blotted Immobilon membranes were stripped and reprobed with an anti-synaptotagmin VI antibody to control for loading. A blot representative of three experiments is shown. C. 50×10^6 permeabilized spermatozoa were incubated with the PLC inhibitor U73122 (15 μM), for 15 min at 37 °C. After that, cells were incubated for an additional 15 min with 0.075 μCi [γ-^{32}P]ATP with no additions (control) or stimulated with 10 μM calcium, 200 nM PMA, or 10 μM PA. Phospholipids were subsequently extracted and analyzed by thin layer chromatography as described in Section 2.8. Each spot was identified by using phospholipid standards. D, Quantification of the spot intensity. The values were normalized with respect to the control (mean ± SEM of 4 independent experiments). Significant increase of PIP_2 and PIP_3 synthesis was assessed by *t*-test for single group mean (*, $P<0.05$).

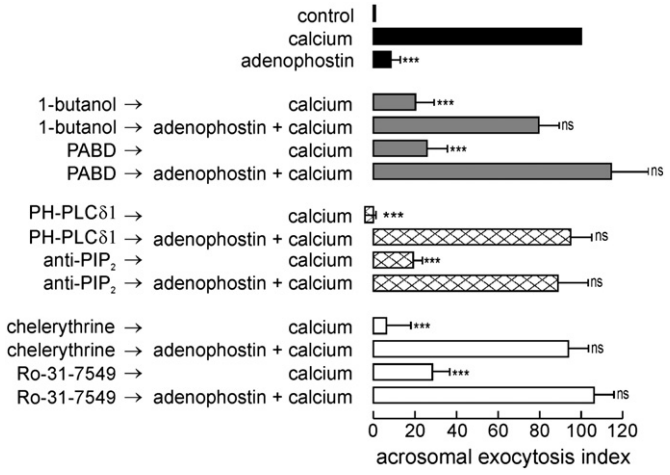

Fig. 6. Adenophostin, an IP_3 receptor agonist, rescues exocytosis impaired by inhibiting PKC or PLD or sequestering PIP_2. SLO-permeabilized human sperm were treated for 15 min at 37 °C with compounds affecting PA availability (gray bars, 0.5% 1-butanol, 10 μg/ml Phosphatidic Acid Binding Domain, PABD), PIP_2 availability (crossed line bars, 10 μg/ml PH-PLCδ1 domain, 20 μg/ml anti-PIP_2 antibody), or PKC activity (white bars, 10 μM chelerythrine, 10 μM Ro-31-7549). The samples were further incubated for 15 min at 37 °C with 10 μM calcium supplemented, when indicated, with 2.5 μM adenophostin. Control conditions are shown as black bars: acrosomal exocytosis in the absence of any stimulation (control), treated with 10 μM calcium, or 2.5 μM adenophostin. Acrosomal exocytosis was evaluated as explained in Section 2.3. The data represent the mean ± SEM from 5 to 10 independent experiments. Dunnett's test was used to compare the means of all groups against the calcium stimulated condition in the absence of inhibitors, and classified as nonsignificant (ns, $P > 0.05$) or significant (***, $P < 0.001$).

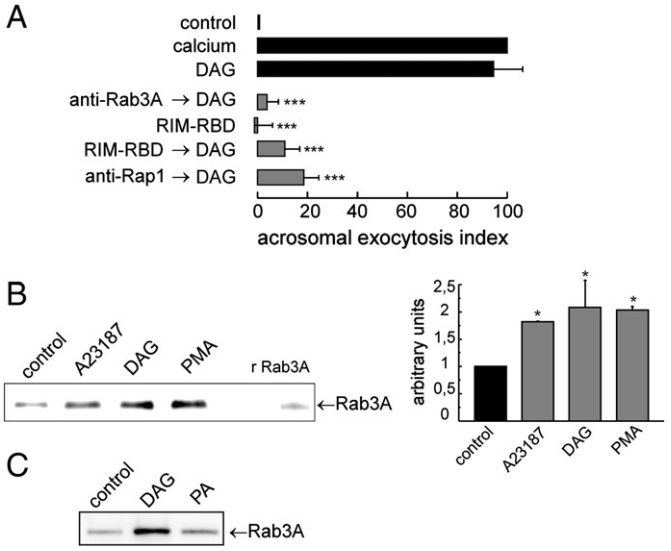

Fig. 7. PMA and DAG stimulate a GTPase exchange factor (GEF) for Rab3A during sperm exocytosis. A, permeabilized spermatozoa were loaded with 10 μg/ml anti-Rab3A antibodies, 5 μg/ml RIM-RBD (which specifically binds active Rab3A) or 20 μg/ml anti-Rap1 antibodies for 15 min at 37 °C. Subsequently, exocytosis was triggered with 10 μM DAG. Control conditions are shown as black bars: acrosomal exocytosis in the absence of any stimulation (control), treated with 10 μM calcium, or 10 μM DAG. Acrosomal exocytosis was evaluated as explained in Section 2.3. The data represent the mean ± SEM from 3 to 7 independent experiments. Dunnett's test was used to compare the means of all groups against the DAG stimulated condition in the absence of inhibitors (***, $P < 0.001$). B, sperm suspensions (30×10^6 cells) were incubated for 15 min at 37 °C with 100 μM 2-APB to prevent protein loss due to exocytosis. Cells were then loaded with permeant Rab3A (100 nM) for an additional 15 min and either were not treated further (control) or were incubated with 10 μM A23187, 10 μM DAG, or 200 nM PMA for 15 min at 37 °C. Cells were disrupted; whole cell lysates were subjected to pull down assays using RIM-RBD-Sepharose beads, and the levels of GTP-bound Rab3A were analyzed by Western blot as described in Section 2.5 of Materials and methods. Recombinant Rab3A used for the pull down assay was used as a control (r Rab3A). A blot representative of three repetitions is shown. *Right*, quantification (carried out with Image J, freeware from National Institutes of Health) is depicted besides the immunoblot as mean ± SEM from all replicates; * significantly different from 1, *t*-test for single group mean. C, 30×10^6 cells processed as described in B, conditions: without any treatment (control) or treated with 10 μM DAG or 10 μM PA. A blot representative of three repetitions is shown.

upstream of PLC. RapGRP/CalDAG is a DAG-sensitive GEF for Rap1 [52]. We tested whether DAG-induced exocytosis involved Rap1. We observed that anti-Rap1 antibodies inhibited DAG-induced exocytosis (Fig. 7A). These observations suggested that DAG may activate the two branches of acrosomal exocytosis.

Therefore, we assessed whether DAG was able to activate Rab3A. To this end, membrane permeant geranylgeranylated Rab3A [32] was incorporated into sperm before challenging with PMA or DAG. The calcium ionophore (A23187) served as a positive control since calcium promotes Rab3A GDP/GTP exchange in sperm [28,53]. Subsequently, we conducted pull down assays using the same Rab3-GTP binding domain of RIM. As shown in Fig. 7B, the Rab3A-GTP level increased 2-fold (quantification in Fig. 7B, *right*) upon treatment with DAG or PMA, confirming that human sperm contain an activity that exchanges GDP for GTP in recombinant Rab3A in response to PMA or DAG. In contrast PA, which does not trigger exocytosis on its own but is absolutely required for secretion, was not able to activate Rab3A (Fig. 7C). We conclude that DAG triggers exocytosis by promoting both PIP_2 production and Rab3A activation.

4. Discussion

Membrane fusion plays a central role in many cell processes. Studies with synthetic membranes and theoretical considerations indicate that the accumulation of lipids characterized by negative curvature such as DAG facilitates fusion [54]. DAG produced enzymatically by phospholipases acting on synthetic membranes induces membrane fusion [55–59]. However, the specific role of lipids in natural membrane fusion is not well established. PMA, a stable analog of DAG, is known to potentiate exocytosis and modulate vesicle fusion kinetics in neurons and endocrine cells. The exact mechanisms underlying the actions of PMA, however, is often not clear, largely because of the diversity of the DAG/PMA receptors involved in the exocytotic process [9].

DAG is produced in human spermatozoa undergoing acrosomal exocytosis in response to progesterone treatment [22] and DAG production depends on a prior activation of Ca^{2+}channels [60]. As previously mentioned, addition of DAG or membrane-permeant phorbol esters triggers acrosomal exocytosis in spermatozoa of different species by a still unknown mechanism [18–21].

This paper focuses on the identification of the signal transduction pathway triggered by PMA/DAG which is not related to the regulation of plasma membrane calcium channels. Using SLO-permeabilized human spermatozoa we demonstrated that PMA/DAG activates a complex pathway leading to the release of intraacrosomal calcium through IP_3-sensitive calcium channels (Fig. 1 and Supplementary Fig. 1). Furthermore, the results showed that SNAP25 and syntaxin are involved in the lipid-triggered acrosome reaction indicating that PMA/DAG-induced exocytosis is a SNARE-dependent membrane fusion process. Additionally, we demonstrated that DAG promotes cis complex disassembly. This mechanism leaves SNAREs in a free monomeric state ready to assemble in loose trans complexes, causing the irreversible docking of the acrosome to the plasma membrane. Moreover, we showed that DAG and calcium share the same pathway, suggesting that this lipid is part of the secretion mechanism triggered by physiological agonists.

We characterized several steps of the signaling pathway stimulated by DAG. The results point to PLD as a key protein in the process. This enzyme is responsible for PA production in most cells. PLD1 participates in outward trafficking of secretory vesicles in neuroendocrine [61], adipocyte [62] and mast cells [63], and in the fusion of secretory granules

with the plasma membrane during regulated exocytosis in neuroendocrine and neural cells [64,65]. On the other hand, PLD activity has been shown to be required for actin polymerization during bovine spermatozoa capacitation [66]. Roldán and Dawes [67] reported that calcium entry into ram sperm cells triggers a late activation of PLD, although they suggested that PLD does not make a substantial contribution to the events leading to exocytosis of the sperm acrosome. Mice lacking PLD1 are viable and fertile, suggesting that PLD1 deficiency can be functionally compensated by PLD2, [68,69] or any of the other signaling enzymes that increase the production or decrease the catabolism of PA, such as DAG kinases and LysoPA acetyltransferases [70]. Our results indicate that PLD1 is present in the acrosomal region of human sperm and that PMA and calcium promote PLD1 enrichment in membranes (Fig. 5B). Furthermore, we observed that these stimuli increased PLD activity (Fig. 5A). Inhibition of the pathway leading to PLD activation was rescued by PA, indicating that this lipid is crucial for acrosomal exocytosis.

For some cell types, PKC has been suggested to be the major cellular activator of PLD. Some studies have shown that PKC can directly activate PLD1 in an ATP-independent manner, while others suggest that a phosphorylation-dependent mechanism is important. PLD1 is phosphorylated by PKCα at residues serine 2, threonine 147 (located in the PX domain) and serine 561 (located in the loop region) and mutation of any of these residues leads to a significant reduction in PMA-induced PLD1 activity [71]. In this work we rescued the calcium- and DAG-induced exocytosis abolished by PKC inhibitors with PA. These experiments led us to conclude that PKC is necessary for PLD activation. Taken together, our results indicate that calcium and DAG promote the PKC-dependent activation of PLD1 and that PA production is critical for sperm acrosomal exocytosis.

It has been shown that PA activates phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) to generate PIP_2 [72]. Synthesis and turnover of the membrane phospholipid PIP_2 are involved in various cellular processes playing a crucial role in cellular signaling [73]. PIP_2 is involved in exocytic and endocytic membrane traffic [74,75], ion channel and transporter function [76], enzyme activation [77], and protein recruitment [78]. Regarding exocytosis, PIP_2 has been shown to regulate fusion both as a fusion restraint, that syntaxin-1 alleviates, and as an essential cofactor that recruits protein priming factors to facilitate SNARE-dependent fusion [12]. Recently, van den Bogaart et al. described that PIP_2 increases 40-fold the calcium affinity of synaptotagmin-1 [79]. Here we provide evidence that PIP_2 is required for calcium- and DAG-triggered exocytosis (Figs. 3 and 4). Additionally, we show that both PMA and calcium activate PIP_2 synthesis in human sperm (Fig. 5C), suggesting the participation of the PI4P5K in the exocytic process.

The results depicted in Fig. 6 show that adenophostin, a potent agonist of IP_3 receptors, rescued calcium-induced exocytosis impaired by inhibiting PKC, PLD or sequestering PIP_2. This observation supports the idea that PIP_2 is hydrolyzed by a PLC to produce IP_3 and DAG, closing a positive feedback loop. In a previous report we have shown that cAMP elicits exocytosis via Epac, a guanine nucleotide exchange factor for the small GTPase Rap1, which in turn activates a PLC [28]. We observed that anti-Rap1 antibodies inhibited DAG-induced exocytosis suggesting that this small GTPase is required for DAG-induced exocytosis. We propose that DAG may activate PLC through RapGRP/CalDAG, which is a DAG-sensitive GEF for Rap1 [52].

Additional data indicate that exocytotic stimuli such as calcium or PMA activate PIP_3 synthesis (Fig. 5C), consistent with results published by Jungnickel et al. [80] in mouse sperm. They demonstrated that ZP3 stimulation leads to the accumulation of PIP_3 in mouse sperm, resulting in the activation of the downstream effector protein kinase, Akt (protein kinase B), and PKCζ. This signaling cascade seems to be essential for the acrosome reaction. Considering that ZP3 induces an increase in DAG [22] and calcium [81] in spermatozoa, both signaling cascades could be operating in a concerted fashion to accomplish exocytosis.

PA and PIP_2 are not able to trigger exocytosis alone. Moreover, the IP_3-sensitive calcium channel agonist, adenophostin, did not trigger exocytosis. According to previous results, acrosome secretion requires not only the release of calcium from intracellular stores, but also the activation of a complex membrane fusion machinery including Rab3A, NSF, SNAREs, complexin and synaptotagmin [14]. To trigger exocytosis, DAG should, in addition to producing IP_3, activate this machinery. Active Rab3A (i.e., loaded with GTP) can trigger acrosomal exocytosis in permeabilized sperm [50]. Moreover, a membrane permeant Rab3A construct induces secretion in non permeabilized sperm [32]. We found that PMA and DAG stimulated the accumulation of Rab3A in the active form in sperm; in contrast PA was ineffective (Fig. 7). Altogether, our results indicate that DAG can activate the continuous production of IP_3 and the Rab3A-dependent activation of the membrane fusion machinery.

On the basis of the results presented here and previous publications from our laboratory we present a model depicting our current thinking on the mechanisms underlying acrosomal exocytosis in Fig. 8. In permeabilized human sperm, calcium triggers exocytosis through a pathway involving cAMP production and Epac activation [82]. These events drive sequential protein–protein and protein–lipid interactions as well as the mobilization of calcium from an intracellular store. The acrosomal exocytosis seems to be organized as a bifurcated pathway, with two separate branches that diverge downstream of cAMP/Epac [28]. One limb starts with Rab3A activation that leads to the fusion protein machinery assembly allowing that the outer acrosomal and plasma membranes become physically attached [83] whereas the other branch drives PIP_2 and IP_3 synthesis with the consequently mobilization of calcium from an intracellular store. The results collected here show that DAG may participate in both branches, activating Rab3A and fostering a positive feedback loop necessary for the continuous production of PIP_2 and IP_3.

In this study we present pieces of evidence suggesting that DAG, PA, and PIP_2 participate in IP_3 production. However, we cannot discard the possibility that lipid remodeling during exocytosis may have some other functions, such as affecting the membrane curvature of the outer acrosomal and/or the plasma membrane. It has been demonstrated that prior to the acrosomal calcium release the granule is profusely swollen, with deep invaginations of the outer acrosomal membrane. The changes in lipid composition occurring upon calcium or DAG stimulation may be implicated in the striking change in membrane topology occurring during the acrosomal exocytosis [83]. We also cannot discard the possibility that lipid remodeling plays a role in the proper assembly of the fusion machinery organizing specific membrane microdomains, providing binding sites for proteins carrying lipid-binding domains. For instance it was recently reported that PA binds to the juxtamembrane domain of the SNARE protein syntaxin-1 and affects membrane fusion [84].

Therefore, we propose that after a calcium influx through plasma membrane channels, DAG, PIP_2, and PA stand as key regulatory lipids that modulate IP_3 production and Rab3A activation. The interplay between lipids and proteins seems to be of outstanding importance for human spermatozoa acrosomal exocytosis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2012.05.001.

Abbreviations

DAG	diacylglycerol
PMA	phorbol 12-myristate 13-acetate;
αPMA	4α-phorbol 12-myristate 13-acetate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLD1	phospholipase D1
PA	phosphatidic acid
PKC	protein kinase C
PLC	phospholipase C
IP_3	inositol 1,4,5-trisphosphate

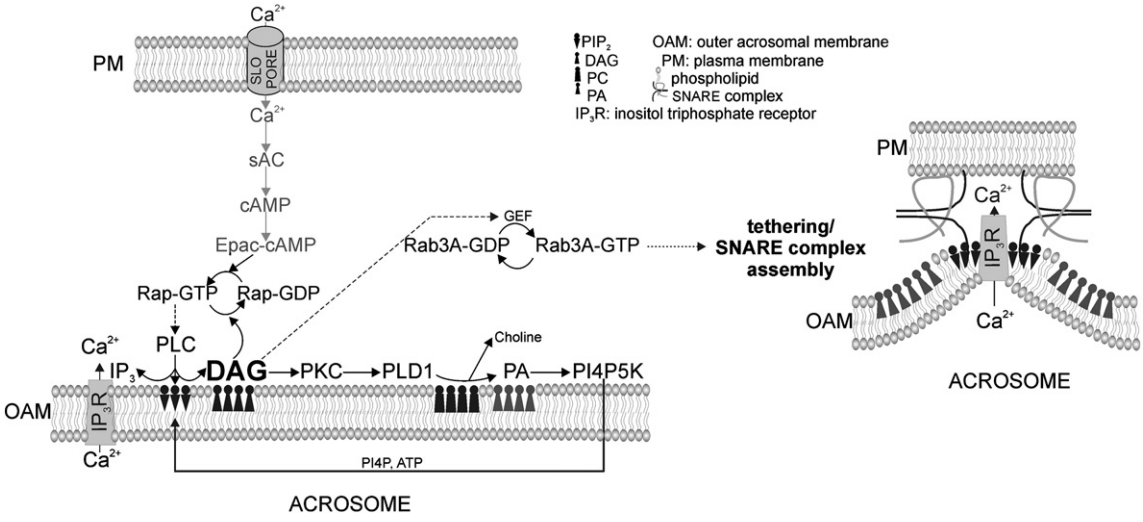

Fig. 8. Working model of the interaction between lipids and proteins involved in the biochemical cascade leading to the acrosomal exocytosis. Calcium or DAG enters the cell from the extracellular milieu through SLO-pores in the plasma membrane (PM). The calcium increase triggers cAMP synthesis likely by a soluble adenylyl cyclase (sAC) and activates Epac, which is an exchange factor for the Rap1 GTPase [28] (gray letters). An effector for active Rap1 is PLCε that can hydrolyze PIP_2 to generate DAG and IP_3. When DAG is added to SLO-permeabilized cells the mechanism described above is bypassed and the lipid activates Rap generating a loop shown in the cartoon (black letters). The results in the present report indicate that DAG causes the PKC-mediated activation of PLD1. PLD1 hydrolyzes phosphatidylcholine (PC) generating choline and phosphatidic acid (PA). The latter activates a phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) which synthesizes PIP_2 from PI4P and ATP closing a positive feedback loop. IP_3 elicits the efflux of calcium from an IP_3-sensitive store (likely the acrosome). Simultaneously, DAG connects with the other branch of the membrane fusion machinery by activating Rab3A, which presumably initiates downstream effects leading to the assembly of trans SNARE complexes. Lipid modifications during the exocytotic stimulus may induce changes in membrane curvature fostering the growing of deep invaginations of the outer acrosomal membrane (PA) and probably recruiting SNARE proteins (PIP_2). SNAREs present in the plasma membrane interact with SNAREs in the protruding edge of cup-shaped invaginations of the outer acrosomal membrane to form trans complexes. Calcium released locally in this ring of apposed membranes would trigger the synaptotagmin-mediated opening of fusion pores; the expansion of which would generate the hybrid vesicles that are released during the acrosome reaction. Solid arrows mean that there is one step between the terms connected, and dashed arrows mean that the number of steps is either unknown or not depicted for simplicity.

VOCC	voltage-operated calcium channel
SOCC	store-operated calcium channel
PI4P5K	phosphatidylinositol 4-phosphate 5-kinase
PI4P	phosphatidylinositol 4-phosphate
SNARE	soluble NSF attachment protein receptors
HTF	human tubal fluid medium
2-APB	2-aminoethoxydiphenylborate
BAPTA	1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis
PBS	phosphate-buffered saline
FITC	fluorescein isothiocyanate
PSA	Pisum sativum agglutinin

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