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Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation

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Abstract

Mammalian spermatozoa should reside in the female reproductive tract for a certain time before gaining the ability to fertilize. During this time, the spermatozoa undergo a series of biochemical processes collectively called capacitation. We recently demonstrated that actin polymerization is a necessary step in the cascade leading to capacitation. We demonstrate here for the first time a role for phospholipase D (PLD) in the induction of actin polymerization and capacitation in spermatozoa. The involvement of PLD is supported by specific inhibition of F-actin formation during sperm capacitation by PLD inhibitors and the stimulation of fast F-actin formation by exogenous PLD or phosphatidic acid (PA). Moreover, PLD activity is enhanced during capacitation before actin polymerization. Protein kinase A (PKA), known to be active in sperm capacitation, and protein kinase C (PKC), involved in the acrosome reaction, can both activate PLD and actin polymerization. We suggest that PKA- and PKC-dependent signal transduction pathways can potentially lead to PLD activation; however, under physiological conditions, actin polymerization depends primarily on PKA activity. Activation of PKA during capacitation causes inactivation of phospholipase C, and as a result, PKC activation is prevented. It appears that PKA activation promotes sperm capacitation whereas early activation of PKC during capacitation would jeopardize this process.

Keywords: Spermatozoa; Capacitation; Actin; PKA; PKC; PLD

Introduction

Mammalian spermatozoa are unable to fertilize an egg immediately after ejaculation. The spermatozoon must first undergo a series of biochemical transformations in the female reproductive tract, collectively called capacitation. Once capacitated, spermatozoa can bind to the zona pellucida of an egg and undergo the acrosome reaction, a process that enables it to penetrate the egg.

There is no clear recognizable marker for the occurrence of capacitation, although several intracellular changes are known to occur, including increases in membrane fluidity, cholesterol efflux, intracellular Ca^{2+} and cAMP concentrations, protein tyrosine phosphorylation, and changes in swimming patterns and chemostatic motility (reviewed in Breitbart, 2002).

Another important process that occurs during sperm capacitation is F-actin formation, which must be dispersed before acrosomal exocytosis (Brener et al., 2003; Spungin et al., 1995). Although factors known to be involved in capacitation including Ca^{2+}, protein kinase A (PKA), and protein tyrosine phosphorylation are important for actin polymerization, the cellular mechanisms which regulate this process are not known. It has been shown in several cell types that phospholipase D (PLD) is involved in the regulation of the actin cytoskeleton (Cross et al., 1996; Kam and Exton, 2001). Although some evidence suggest that PLD activity is confined to the cytoskeletal fraction and may be involved in actin cytoskeleton rearrangement in vivo, the regulatory mechanism has remained obscure. The actin cytoskeleton is a highly dynamic network composed of actin polymers and a large variety of associated proteins (Glenney et al., 1982; Lebart et al., 1993). In recent reports, PLD activity has been found in the detergent-insoluble fraction of HL-60 and U937 cells (Hodgkin et al., 1999; Iyer and Kusner, 1999) demonstrating the possibility of in vivo interaction between PLD and actin-based cytoskeleton. PLD is a ubiquitous enzyme widely distributed in various mammalian cells including spermatozoa (Garbi et al., 2000). The PLD1 isoform is regulated by protein kinase C (PKC) and small G proteins of the ADP-

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ribosylation factor (ARF) and Rho families (Exton, 1999; Frohman et al., 1999; Houle and Bourgoin, 1999; Liscovitch et al., 2000). Although the regulation of PLD has been studied extensively, its cellular role is largely unclear. In bovine sperm, PLD1 is localized in the acrosomal region suggesting a possible role in the process of capacitation and in the acrosome reaction (Garbi et al., 2000). This PLD1 co-immunoprecipitates with PKCα and the complex decomposes when the sperm are treated with PKC activators such as phorbol myristate acetate (PMA) or lysophosphatidic acid (LPA) (Garbi et al., 2000). It was shown that addition of LPA to fibroblasts activates PLD and causes an increase both in phosphatidic acid (PA) and in the amount of filamentous actin (Ha et al., 1994). Cross et al. (1996) showed that LPA increases the level of PA and F-actin in aortic endothelial cells, and these effects are reduced by butan-1-ol but not by butan-2-ol. They also concluded that PLD plays a role in actin rearrangement in these cells. In the present study, we demonstrate for the first time a role for PLD in F-actin formation during sperm capacitation. We show that PLD-dependent actin polymerization can be induced by activating PKA and PKC, and the crosstalk between these two enzymes is described.

Materials and methods

Materials

Phorbol myristoyl acetate (PMA), BAPTA-AM, dibutyryl cAMP, U73122, and FITC-phalloidin, *N*-acetyl-D-sphingosine (C_2-ceramide), phosphatidic acid (PA), lysophosphatidic acid (LPA), H-89, genestein, and streptomyces chromofuscus-PLD were purchased from Sigma. Bisidolylmaleimide I (GF) and the Ca^{2+} ionophore, A23187, were obtained from Cal-Biochem.

Sperm preparation

Ejaculated bovine sperm provided by Israel Breeders Service (Hasherut Breeder Service, Hafetz Haim, Israel) were collected using an artificial vagina and diluted (1:1, v/v) in NKM medium consisting of NaCl (110 mM), KCl (5 mM), and 3-[*N*-morpholino]propanesulfonic acid (10 mM), pH 7.4. The cells were washed in NKM medium by three centrifugations at 780 × *g* for 10 min and the final pellets were resuspended in NKM with the sperm concentration adjusted to $1-3 \times 10^9$ cells/ml. Only sperm cells with good motility (>70% motile cells) were used in the experiments.

Sperm capacitation

In vitro capacitation of bull sperm was induced by the method of Parrish et al. (1988). Briefly, washed sperm pellets were resuspended to a final concentration of 10^8 cells/ml in glucose-free TALP medium containing (in mM): 100 NaCl, 3.1 KCl, 1.5 $MgCl_2$, 25 $NaHCO_3$, 0.29 KH_2PO_4, 21.6 sodium lactate, 0.1 sodium pyruvate, 2 $CaCl_2$, 20 HEPES pH 7.4, and 50 μg/ml BSA, 10 U/ml penicillin, and 20 μg/ml heparin. The cells were incubated in this capacitation medium for 4 h at 39°C with 5% CO_2.

Fluorescence staining of actin filaments

Samples of cells were spread on microscope slides. After air drying, sperm were fixed in formaldehyde (5%) for 10 min, washed with 25 mM Tris-buffered saline pH 7.6 (TBS), then dipped in 0.5% Triton X-100 for 30 min, washed three times at 5 min intervals in distilled water, air dried, then incubated with FITC-Phalloidin (3 μM in TBS) for 1 h, washed four times with H_2O at 10-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Lab., Richmond, CA). Actin polymerization was determined by fluorescence microscopy and the fluorescence intensity was determined quantitatively using the MetaMorph "Image J" and Adobe PhotoShop processing software.

Measurement of PLD activity

The assay was performed as previously described (Hess et al., 1997) with minor modifications. Briefly, sperm suspensions were labeled with 5 μCi [^{3}H]-palmitic acid/ml for 1 h at 37°C, washed by centrifugation at 6750 × *g* for 10 min at room temperature, and resuspended in mTALP with 0.5% butan-1-ol. After different treatments, reactions were stopped by the addition of chloroform/methanol (1:2 v/v) and lipids were then extracted as described by Bligh and Dyer (1959). The lower phase was dried under N_2 and resuspended in 50 μl chloroform/methanol (1:2 v/v). Lipids were separated by thin layer chromatography (tlc) on silica-gel 60 F_{254}-coated plates (0.25-mm thickness; Merck-BDH). The plates were developed in the upper phase of a solvent system ethylacetate/2,2,4-trimethylpentane/acetic acid/H_2O (110:50:20:100 v/v). Lipids spots were visualized by exposing the tlc plates to iodine vapor and identified by comparison with PtdBut standard run in parallel on the same plate. The spots in each lane were scraped into scintillation vials containing 4 ml scintillation fluid and samples were counted in a TRI-CARB liquid scintillation counter. The results presented as percent of total lipid cpm incorporated into PtdBut.

Subcellular fractionation

Sperm cells were fractionated to separate cytosol, membranes, and particulate fraction proteins. Cells (1.5×10^9) were resuspended in 20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 1 mM benzamidine, 1 mM Na_3VO_4, 10% glycerol, 25 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM PMSF (homogenization buffer), and then sonicated (3 × 10 s pulses, power setting 4) with a Vibra Cell, Sonics (Sonics and Materials Inc., Danbury, CT) material sonicator. The homogenate was centrifuged for 10

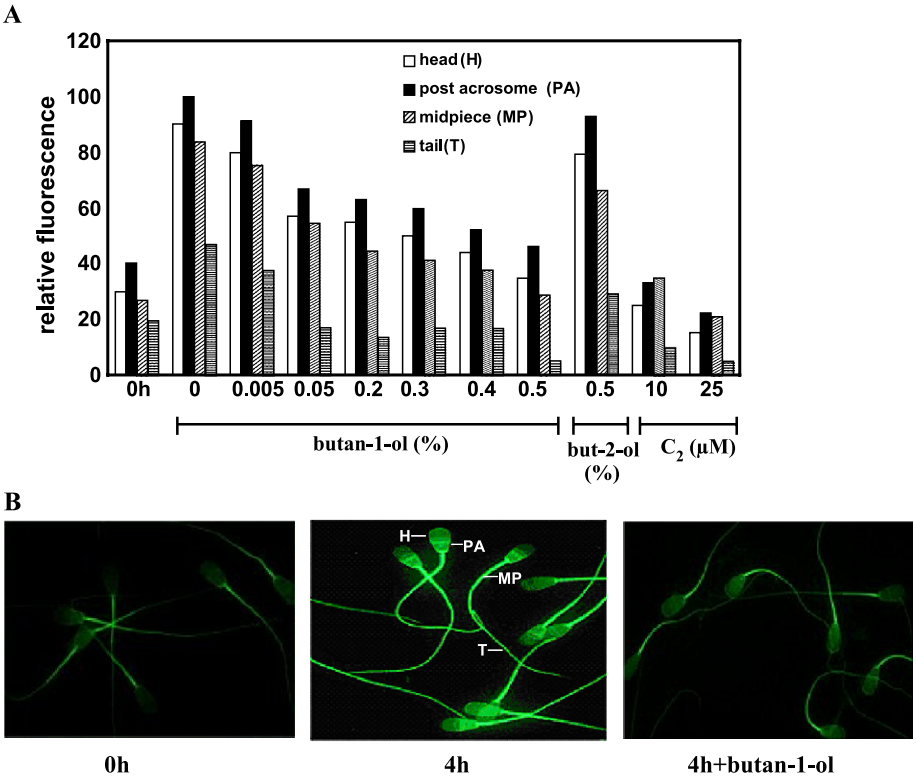

Fig. 1. Reduction of actin polymerization during capacitation by PLD inhibition. (A) Bovine spermatozoa incubated under capacitation conditions in the presence of increased concentrations of butan-1-ol, C_2-ceramide (C_2), or 0.5% (v/v) butan-2-ol (but-2-ol). After 4 h of incubation, the cells were stained with FITC-phalloidin photographed under fluorescence microscope and analyzed for fluorescence intensity in the sperm head (H), post acrosome region (PA), midpiece (MP), and rest of the tail (T). 0 h = zero time control. (B) Representative picture of sperm F-actin at zero time (0 h), after 4-h capacitation (4 h), or after 4-h capacitation in the presence of 0.5% (v/v) butan-1-ol. Magnification: ×1500.

min at 10,000 × *g* for pelleting the particulate fraction containing head and tail fragments. The resulting supernatant was centrifuged at 100,000 × *g* (60 min, 4°C) for recovery of the cytosolic fraction (supernatant) and the membrane fraction (pellet). The cytosolic fraction was concentrated to at least 1/10 of the original volume using

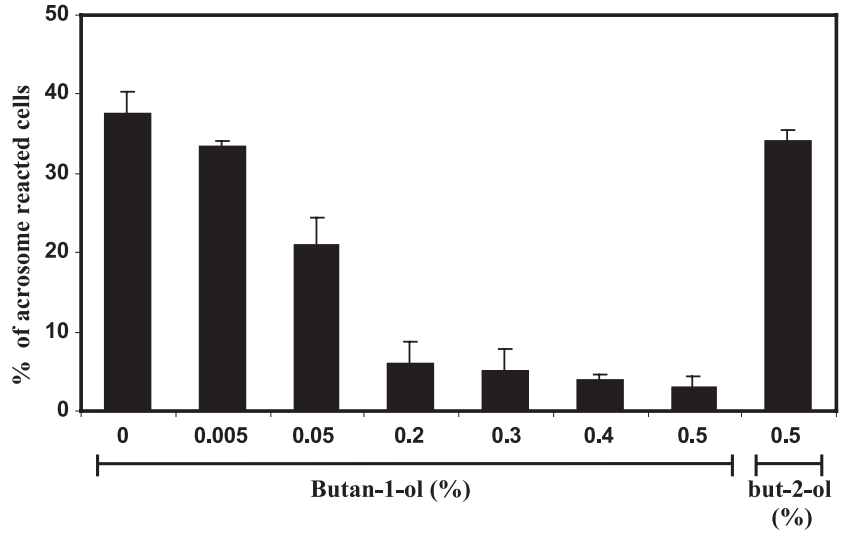

Fig. 2. Reduction of acrosome reaction rate by PLD inhibition. Bovine spermatozoa were capacitated for 4 h in the presence of increasing concentrations of butan-1-ol before induction of acrosome reaction using Ca^{2+}-ionophore 23187. After 20-min incubation, the cells were stained with Coomassie blue and the percentage of acrosome reacted cells were determined. The data represent the mean ± SE of three experiments performed in duplicates.

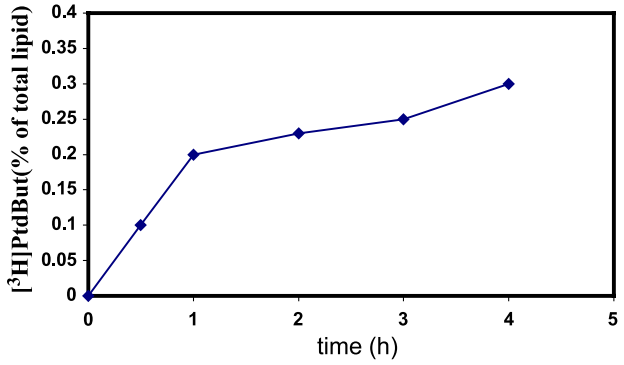

Fig. 3. Induction of PLD activity during capacitation. Bovine sperm were incubated in capacitation medium, and at the indicated times, samples were taken out for the determination of PLD activity as described in Materials and methods. Zero time activity of 0.07% total lipids was subtracted from each point.

a microconcentrator 30 (Amicon, Lexington, MA). The membrane fraction was resuspended in homogenization buffer supplemented with 0.6% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Rush et al., 1992). The particulate fraction was solubilized with SDS-lysis buffer. Protein concentration of the cytosolic and membrane fractions was determined by Bradford's method (Bradford, 1976) using Bio-Rad reagents. For immunoblot analysis, the cell lysates were boiled for 5 min in SDS-PAGE sample buffer (Laemmli, 1970) and separated on a 7.5% SDS-polyacrylamide gel.

Immunoblot analysis

For immunoblotting, proteins derived from equivalent cell numbers were separated on 7.5% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes (200 mA; 1 h) using a buffer composed of 25 mM Tris (pH 8.2), 192 mM glycine, and 20% methanol. For Western blotting, nitrocellulose membranes were blocked with 5% BSA in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. The PKCα isoform and PLD were immunodetected using the following antibodies: rabbit polyclonal anti-PKCα (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 and rabbit polyclonal anti-PLD1 (generated against a peptide corresponding to amino acid residues 675–688 of human PLD1 (Czarny et al., 1999) and kindly provided by Prof. M. Liscovitch of The Weizmann Institute of Science) diluted 1:1000. The membranes were incubated overnight at 4 °C with the appropriate primary antibody. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-linked secondary antibody (Jackson Laboratories, West Grove, PA) diluted 1:10,000 in TBST. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK). Specificity of the antibodies was confirmed by preabsorbing the antibodies with 10 μg of their peptide antigens for 1 h before incubating the antibodies with the

Table 1
Induction of actin polymerization by exogenous phosphatidic acid (PA)

	PA (μg/ml)					PLD (U/ml)
	0	0.1	1.0	3.0	10.0	5.0
Relative fluorescence	34 ± 2	60 ± 3	82 ± 3	132 ± 8	122 ± 5	99 ± 6

Bovine spermatozoa incubated in capacitation medium for 5 min in the presence of increased PA concentrations or for 10 min with 5 U/ml PLD. The data represent the mean ± SE of two experiments. The amount of F-actin was determined in the sperm head by measuring the fluorescence intensity after staining with FITC-phalloidin.

Table 2
Actin polymerization induced by PA is not affected by inhibition of PLD, PKA, or PKC activities

Treatment	Relative fluorescence	
	PA (3 min)	Capacitation (4 h)
Control	108 ± 5	96 ± 1
Butan-1-ol (0.5%)	95 ± 3	36 ± 2
H-89 (50 μM)	97 ± 4	39 ± 5
GF (0.1 nM)	93 ± 3	68 ± 5

Bovine spermatozoa were preincubated for 10 min with 0.5% (v/v) butan-1-ol, 50 μM H-89 (PKA inhibitor), or 0.1 nM GF (PKC inhibitor). At the end of this preincubation, 3 μg/ml PA was added to the cells, and the amount of F-actin was determined in the sperm head after 3 min of incubation. Control cells incubated for 4 h of full capacitation showed reduction of F-actin by the three inhibitors. The fluorescence intensity at zero time is 30 units. The data represent the mean ± SE of three experiments performed in duplicates.

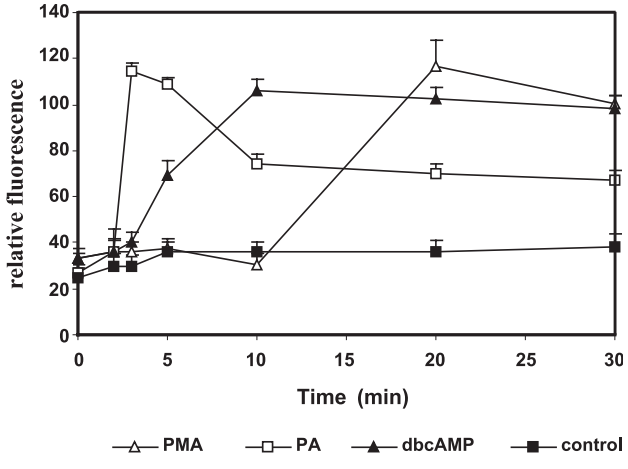

Fig. 4. Time curves of actin polymerization induced by activating PKC, PKA, or by PA. Bovine spermatozoa incubated in full capacitation medium in the presence of PMA or PA, or in HCO_3^--deficient capacitation medium containing dbcAMP. At the indicated times, samples were stained with FITC-phalloidin and fluorescence intensity in the sperm head was determined. Activators were used at the following concentrations: 100 ng/ml PMA, 3 μg/ml PA, 1 mM dbcAMP.

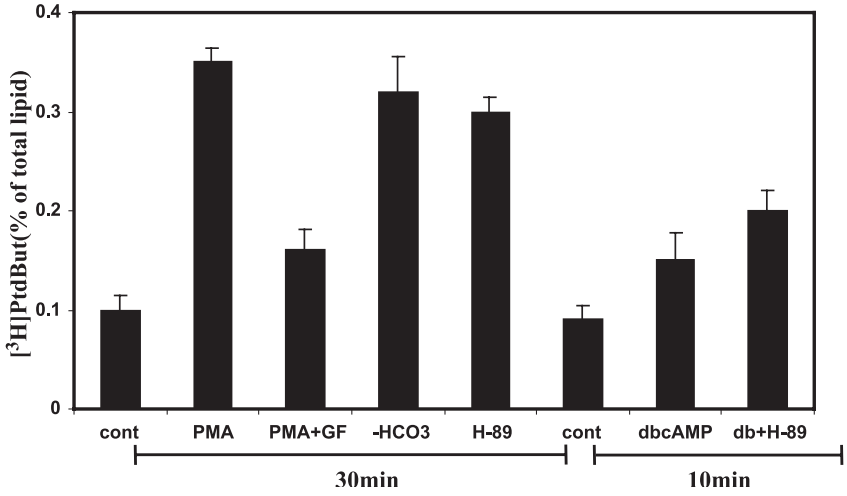

Fig. 5. Activation of PLD by PKC and PKA activation. Bovine sperm were incubated in TALP medium or in HCO_3^--deficient TALP medium ($-HCO_3^-$) under the indicated conditions. At the end of the incubation period, PLD activity was determined as described in Materials and methods. The concentrations used are 100 ng/ml PMA, 0.1 nM GF, 50 μM H89, and 1 mM dbcAMP. The value for 10-min H-89 control is 0.19 ± 0.02%. The data represent the mean ± SE of three experiments. Zero time activity of 0.07% total lipids was subtracted from each point.

membrane. Quantitation of Western blots was performed using a laser densitometer.

Results

Reduction of PLD activity inhibits F-actin formation

We have recently shown that actin polymerization occurs during mammalian sperm capacitation (Brener et al., 2003). Incubation of bovine sperm under conditions that promote capacitation in the presence of increasing concentrations of the PLD inhibitor, butan-1-ol, revealed a dose-dependent inhibition of actin polymerization (Fig. 1A). At 0.5% (v/v) butan-1-ol, there is almost complete inhibition of capacitation-induced actin polymerization whereas only a small effect was seen in the presence of 0.5% (v/v) butan-2-ol which does not affect PLD activity. Butan-1-ol is widely used to identify PLD activity because it can replace water as PLD substrate and the phosphatidic acid (PA), which is the

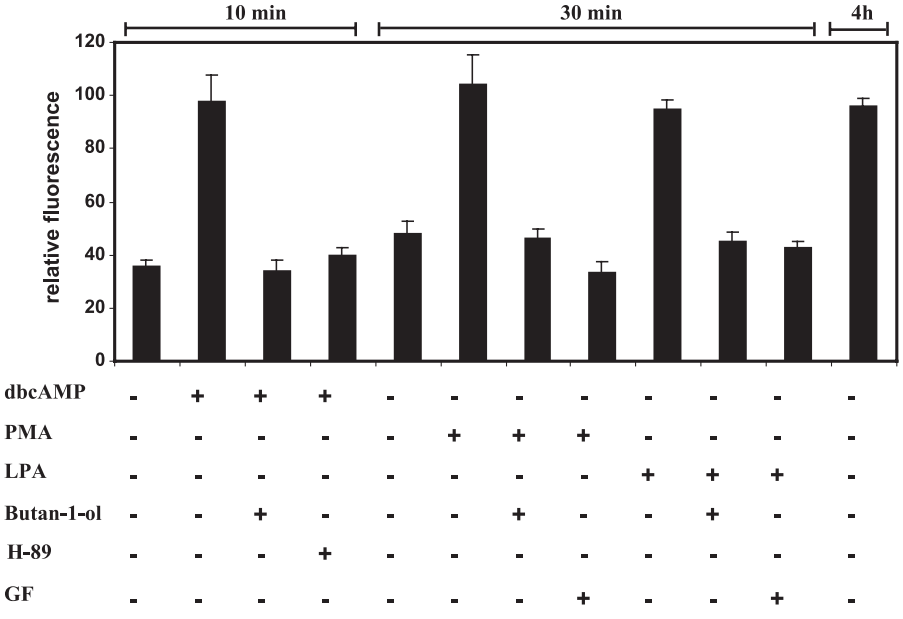

Fig. 6. Effects of PKA and PKC activators and inhibitors on actin polymerization. Spermatozoa were incubated for the indicated times in the presence of 1 mM dbcAMP (db, activator of PKA), 100 ng/ml PMA, or 1 μM LPA (activators of PKC), 0.5% (v/v) butan-1-ol (but, inhibitor of PLD), 50 μM H-89 (inhibitor of PKA), or 0.1 nM GF (PKC inhibitor). At the end of the incubation, the sperm heads were analyzed for F-actin content using FITC-phalloidin. The data represent the mean ± SE of three experiments performed in duplicates.

active product of phospholipids hydrolysis (Fang et al., 2001), undergo trans-phosphatidylation to phosphatidylbutanol by the PLD. This does not occur with butan-2-ol; therefore, this reagent does not block PLD activity. The C_2-ceramide, *N*-acetyl-D-sphingosine at concentrations known to inhibit PLD (Gomez-Munoz et al., 1995; Jones and Murray, 1995; Nakamura et al., 1996) reduced F-actin formation (Fig. 1A) and caused 80% inhibition of PLD activity in bovine sperm.

In the study, we analyzed actin polymerization in various regions of the sperm. Polymerization is enhanced similarly in four different regions of the sperm including the head, post acrosome, midpiece, and the rest of the tail (Figs. 1A and B). Separate analysis of each region was performed in each of the experiments described in this study, but because no significant differences were observed among the four regions, the presented data describe changes in actin polymerization in the sperm head only.

Determination of acrosomal exocytosis as an additional marker for sperm capacitation also revealed a dose-dependent inhibition by increased concentrations of butan-1-ol but not by butan-2-ol (Fig. 2). F-actin formation is gradually enhanced during sperm capacitation (Brener et al., 2003, Fig. 9). Here we showed that PLD activity is also enhanced during capacitation before actin polymerization (Fig. 3). The correlation between these two activities supports our notion regarding the involvement of PLD in the process of actin polymerization. To further support the role of PLD in actin polymerization, we measured F-actin formation after adding PLD to sperm cells. It was shown in other cell types that exogenous PLD can induce actin polymerization (Ha and Exton, 1993). Within 10 min of incubation with exogenous PLD, we can see a high level of F-actin, which is comparable to the level found after 4 h of sperm capacitation (Table 1). Addition of phosphatidic acid (PA), the product of phospholipid hydrolysis by PLD, to the cells also stimulated actin polymerization in a dose-dependent manner within 5 min (Table 1). In fact, the degree of F-actin formation by adding 3 μg/ml PA for 5 min of incubation is 30% higher than the amount observed after 4 h of incubation under capacitation conditions. This suggests that the amount of intracellular PA might be an important factor in the regulation of F-actin formation. Moreover, the fact that the stimulation of actin polymerization by PA is not significantly affected by butan-1-ol (Table 2) further indicates that this inhibitor specifically blocks PLD activity.

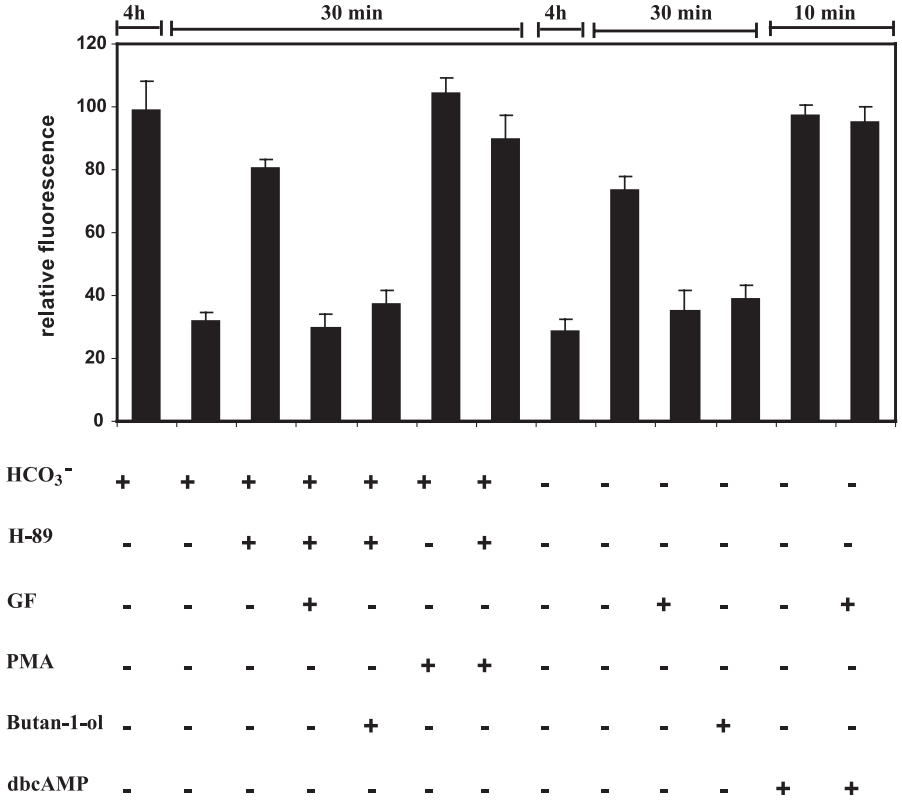

Fig. 7. Actin polymerization induced by dbcAMP, H-89, or in HCO_3^--deficient medium. Spermatozoa were incubated for 30 min under the indicated conditions. $-HCO_3$ represent capacitation medium without added HCO_3^-. $+HCO_3^-$ represents regular capacitation medium. The concentrations used were 0.5% (v/v) butan-1-ol, 50 μM H-89, 100 ng/ml PMA, and 1 mM dbcAMP. Additional control values of relative fluorescence for the 30-min incubation: $+HCO_3^-$ + GF = 30 ± 1.1%; $+HCO_3$ + but-1-ol = 28 ± 1.2%; $-HCO_3$ + PMA = 76 ± 2.0%. The data represent the mean ± SE of three experiments performed in duplicates.

Role of protein kinases in PLD activation

In a previous report, we show that PLD1 and protein kinase Cα (PKCα) co-immunoprecipitate from bovine sperm extract (Garbi et al., 2000). Moreover, we show that activation of PKC by phorbol ester (PMA) or by lysophosphatidic acid (LPA) causes fast translocation of PKCα to the plasma membrane and PLD1 to the particulate fraction, probably to the outer acrosomal membrane (Garbi et al., 2000). Here we showed that PMA induced fast actin polymerization which reached its maximal rate after 20 min of incubation (Fig. 4). Another kinase known to

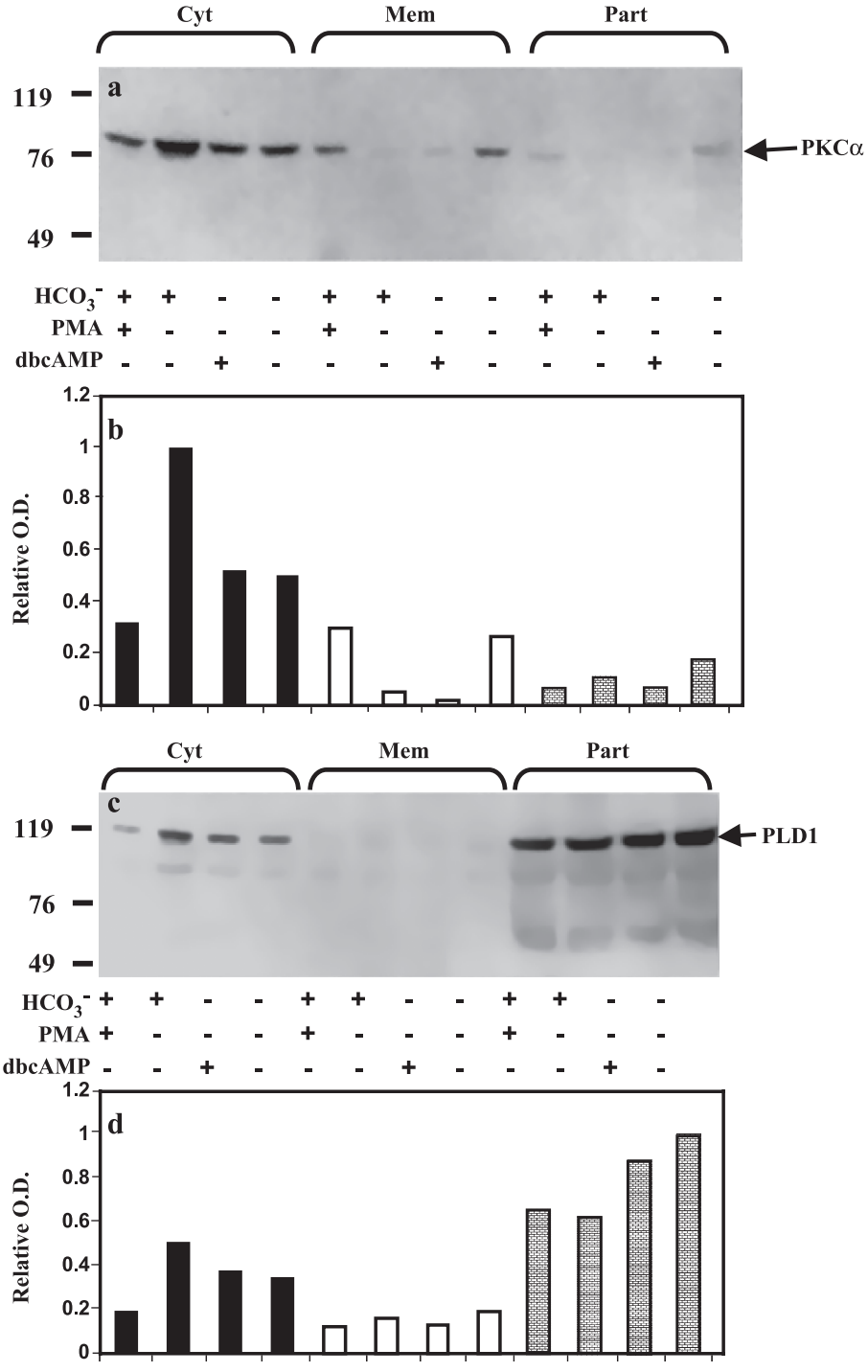

Fig. 8. Subcellular redistribution of PKCα and PLD1. Sperm cells were incubated in regular capacitation medium (M+), in HCO_3^--deficient capacitation medium (M−), in the presence of 100 ng/ml PMA (P), or 1 mM dbcAMP (db) for 10 min. Cells were homogenized and fractionated into cytosol (Cyt), plasma membrane (Mem), and particulate (Part) fractions as described in Materials and methods. (a) Protein kinase Cα-immunodetection. (b) Densitometric analysis of PKCα. (c) Phospholipase D1-immunodetection. (d) Densitometric analysis of PLD1. The blots shown are representative of three separate experiments.

participate in sperm capacitation is the cAMP-dependent protein kinase A (PKA) (Visconti et al., 1995). Sperm incubation for a short time in the presence of the permeable PKA activator, dibutyryl-cAMP (dbcAMP), induces maximal actin polymerization within 10 min (Fig. 4). Addition of phosphatidic acid (PA) to the cells revealed maximal F-actin formation within 3 min of incubation (Fig. 4). Actin polymerization induced during capacitation is only 40% inhibited by low concentrations of the specific PKC inhibitor, bisindolylmaleimide (GF), and almost completely blocked by the PKA inhibitor H-89 (Table 2). However, the stimulatory effect of PA on F-actin formation is not significantly affected by these inhibitors or by butan-1-ol (Table 2), indicating that PKC or PKA activation during capacitation leads to PLD activation and actin polymerization. Treatment of cells with PMA or dbcAMP revealed activation of PLD, indicating the involvement of PKC and PKA in PLD activation (Fig. 5). This idea is further supported by showing almost complete inhibition of PMA or dbcAMP-induced actin polymerization by butan-1-ol, indicating the involvement of PLD in PKC- and PKA-dependent F-actin formation (Fig. 6). LPA, which also activates PKC (Garbi et al., 2000), behaves exactly as PMA in the various treatments (Fig. 6).

Crosstalk between PKA and PKC

To this point we showed that sperm incubation for 4 h in the presence of the PKA inhibitor H-89 or the PKC inhibitor GF caused inhibition of the capacitation-dependent actin polymerization (Table 2). To further investigate the relationship between PKA and PKC, we have tried to locate these enzymes in the cascade leading to F-actin formation. As a first approach to answer this question, we asked if inhibition of PKA would affect actin polymerization induced by activation of PKC and vice versa. We found that the effect of the PKA activator dbcAMP on actin polymerization was not inhibited by the PKC inhibitor GF, and the stimulatory effect of the PKC activator PMA was not affected by the PKA inhibitor H-89 (Fig. 7). Thus, we assumed that PKC and PKA stimulate actin polymerization in two independent pathways.

To further support these data we prevented PKA activation by omitting HCO_3^- from the incubation medium, which is known to activate the soluble adenylyl cyclase (sAC) found in spermatozoa (Chen et al., 2000). In absence of cAMP, PKA is not activated. The data in Fig. 7 revealed stimulation of actin polymerization within 30 min of incubation in HCO_3^--deficient medium. We found that this stimulation is completely blocked by GF (Fig. 7), indicating activation of PKC under these conditions. Inhibition of PKA by h-89 in cells incubated in medium containing HCO_3^- revealed high stimulation of actin polymerization as well, which is also blocked by GF (Fig. 7), supporting our conclusions regarding PKC activation under conditions by which PKA is inhibited. Furthermore, PMA does not increase actin polymerization in HCO_3^--deficient medium (Fig. 7) because PKC is already activated. Under conditions by which PKA activity is inhibited (in HCO_3^--deficient medium or in the presence of H-89) or when PKC is activated by PMA, PLD is activated (Fig. 5) indicating the activation of PKC and PLD when PKA is blocked.

Another approach to determine the relationship between PKC and PKA is to examine direct activation of PKC. Activation of PKC can be determined by following its translocation from the cytosol to the cell plasma membrane (Lax et al., 1997). Thus, we examined whether treatment of cells with dbcAMP would affect PKC translocation to the plasma membrane. We found that incubation of cells in HCO_3^--deficient medium caused significant translocation of PKCα from the cytosol to the plasma membrane fraction, comparable to the PMA effect (Figs. 8a and b). This effect was not further enhanced by adding PMA to HCO_3^--deficient medium (not shown). The translocation of PKC is significantly inhibited by including dbcAMP in the HCO_3^--deficient incubation medium (Figs. 8A and B) and no effect is seen in medium containing HCO_3^- (not shown). Stimulation of PKCα translocation was also induced by treating the cells with the PKA inhibitor, H-89 (not shown). Thus, these data demonstrate that PKC is activated under conditions by which PKA is inhibited.

Cells incubated with dbcAMP or in HCO_3^--deficient medium conditions by which PKCα is translocated, there is a parallel translocation of PLD1 from the cytosol to the particulate fraction (Figs. 8c and d). The amount of PLD1 in PMA or dbcAMP-treated cells or in cells incubated in HCO_3^--deficient medium is significantly decreased in the cytosol and parallel increase in the particulate fraction is observed in cells treated with dcAMP or in HCO_3^--deficient medium, but not in PMA-treated cells (Fig. 8D). It is not clear to us why PMA gives such a different response, although in our previous work we showed that PMA or

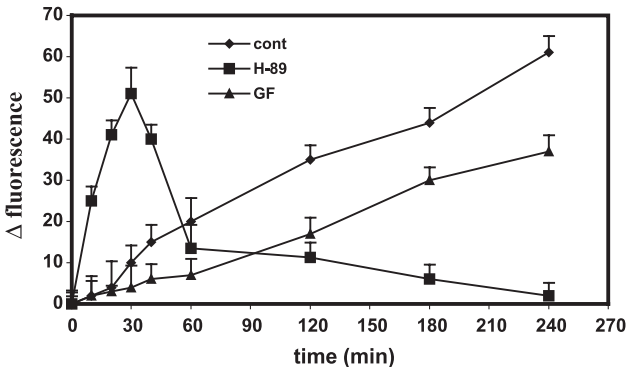

Fig. 9. Time curves of actin polymerization in the presence of PKA or PKC inhibitors. Spermatozoa were incubated in capacitation medium (cont.) or in the presence of 50 μM H-89 or 0.1 nM GF. At the indicated times, samples were taken out and stained with FITC-phalloidin for the determination of F-actin. Data represent the mean ± SE of two experiments performed in duplicates.

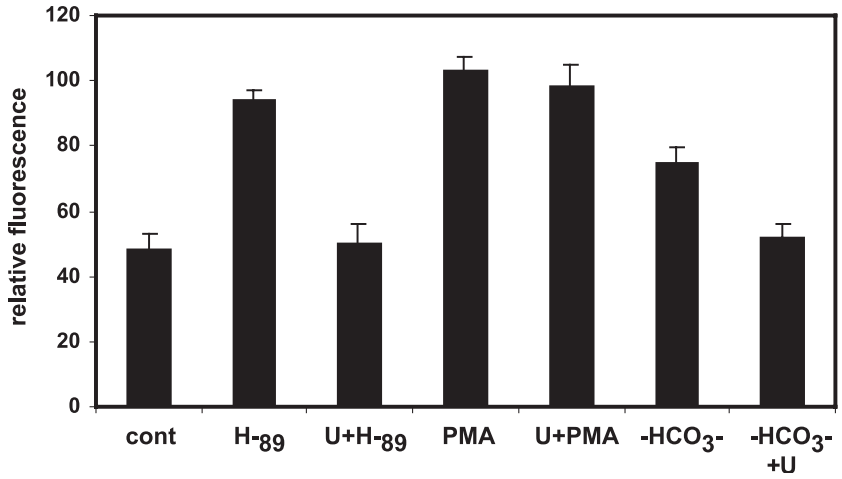

Fig. 10. Effect of phospholipase C inhibition on actin polymerization. Spermatozoa were incubated for 30 min in capacitation medium or in HCO_3^--deficient capacitation medium ($-HCO_3^-$) in the presence of PKA inhibitor (50 μM H-89), PLC inhibitor (1 μM, U73122) (U), or PKC activator (100 ng/ml PMA). The fluorescence intensity of F-actin stained with FITC-phalloidin was determined in the sperm head. Data represent the mean ± SE of two experiments performed in duplicates.

LPA caused significant increase in PLD1 in the particulate fraction (Garbi et al., 2000).

The kinetics of actin polymerization induced by PKC or PKA alone or by both can be seen in Fig. 9. Inhibition of PKA by H-89 allowed PKC activation leading to maximal actin polymerization after 30 min of incubation followed by F-actin depolymerization. At this time, in the control or when PKC is inhibited, there is only a slight increase in actin polymerization which is gradually increased up to 4 h. The rate of actin polymerization at the end of the capacitation time is about 40% inhibited by GF and completely reduced by H-89.

To explain how inhibition of PKA activates PKC, we assumed that PKA inhibits PLC activity resulting in the reduction of diacylglycerol (DAG), which is known to activate PKC. It is shown in Fig. 10 that inhibition of PLC activity by U73122 resulted in the complete inhibition of actin polymerization induced by H-89 or in HCO_3^--deficient medium. This PLC inhibitor did not affect PKC-dependent actin polymerization induced by PMA, indicating the specificity of U73122 inhibition of PLC and not of other steps leading to actin polymerization. In the presence of PMA, there is no need for PLC activity to form DAG.

Discussion

In this study, we demonstrated for the first time a role for PLD in the mechanism leading to F-actin formation during sperm capacitation. In a recent study, we showed that PLD1 is present in bovine sperm and localized mainly in the acrosomal region of the sperm head (Garbi et al., 2000), suggesting its possible involvement in acrosomal exocytosis. It was shown elsewhere that PLD1 activity is required for actin fiber formation in fibroblasts (Kam and Exton, 2001) and is a key factor for exocytotic process in neuroendocrine cells (Vitale et al., 2001). We demonstrated elsewhere that F-actin formation is a necessary event in sperm capacitation and acrosomal exocytosis (Brener et al., 2003; Spungin et al., 1995). In the present study, we showed that PLD activity is required for F-actin formation. Our conclusion is supported by the following data: first, actin polymerization is strongly inhibited by the PLD-specific inhibitors butan-1-ol and C_2-ceramide, but not by butan-2-ol, which does not block PLD activity. Butan-1-ol but not butan-2-ol also inhibits acrosomal exocytosis, which was used as a marker for the occurrence of capacitation. Second, exogenous PLD caused fast actin polymerization. Third, PA, which is the final product of PLD activity, caused a very fast increase in polymerized actin in a dose-dependent matter. This effect of PA is not significantly affected by the PLD inhibitor butan-1-ol, suggesting the high specificity of this inhibitor for PLD inhibition. Fourth, PLD activity is enhanced during capacitation before the elevation of F-actin.

In other cell types, it is known that the small GTPases ADP-ribosylation factor (ARF) and Rho families directly activate PLD1 in vitro (Exton, 1999). The PLD1 isoform is present in bovine sperm (Garbi et al., 2000) and F-actin formation induced during sperm capacitation is blocked by inhibiting Rho using C3 exoenzyme (Brener et al., 2003) or by inhibiting ARF by brefeldin (our unpublished data). These data further support our hypothesis regarding the involvement of PLD in F-actin formation in spermatozoa.

Fast actin polymerization and PLD activation were also induced by activating PKA or PKC (Figs. 4 and 5) and F-actin formation during capacitation is inhibited by PKA or PKC blockers (Table 2). Actin polymerization induced by short time activation of PKA or PKC is completely blocked by butan-1-ol (Fig. 6), whereas the polymerization induced by exogenous PA is not significantly affected by PLD, PKA, or PKC inhibitors (Table 2). These data indicate that activation of PKA or PKC can lead to the activation of

PLD. PKC is known to be involved in the activation of PLD (Exton, 1998) but there is only limited information regarding the activation of PLD by PKA activation (Ginsberg et al., 1997; Mamoon et al., 1999). In bovine sperm, we showed that PKCα and PLD1 coexist as a complex which decomposes after PKC activation with PMA or LPA (Garbi et al., 2000). Here we showed that PMAs stimulate PLD activity as well as actin polymerization, which is inhibited by PKC or PLD inhibitors indicating the involvement of PKC in PLD activation.

The fact that the PKA inhibitor, H-89, completely blocked F-actin formation after 4-h capacitation while PKC inhibition only caused partial inhibition indicated that PKA is obligatory for actin polymerization during sperm capacitation. This is further supported by the fact that 0.1 nM GF does not affect F-actin formation induced by dbcAMP (Fig. 7). If so, it is unclear why PKA inhibition by H-89 does not block the stimulatory effect of PMA on actin polymerization (Fig. 7), indicating that there is no need for PKA activity under these conditions.

Moreover, we found that by preventing PKA activation using H-89 or HCO_3^--deficient medium, actin polymerization is stimulated after 30 min of incubation (Figs. 7 and 9). These stimulatory effects are both inhibited by 0.1 nM GF (Fig. 7) indicating that PKC is activated under these conditions. To further support the activation of PKC under these conditions, we followed PKCα translocation and it is clearly shown that in HCO_3^--deficient medium PKCα is translocated from the cytosol to the plasma membrane in a comparable way to the PMA effect and this translocation is blocked by dbcAMP (Fig. 8). This translocation indicates that PKCα is activated after a short time of incubation in HCO_3^--deficient medium but not when PKA is activated by dbcAMP (Fig. 8). Moreover, F-actin formation in HCO_3^--deficient medium or in the presence of H-89 is inhibited by GF or by butan-1-ol (Fig. 7) and PLD is activated under these conditions (Fig. 5), indicating the activation of PKC and PLD under these conditions. It was interesting to find that in HCO_3^--deficient medium conditions by which PKCα is translocated, PLD1 is also translocated from the cytosol to the particulate fraction (Fig. 8) as occurred after PMA or LPA treatment (Fig. 8 and Garbi et al., 2000). In addition, dbcAMP, which inhibits PKCα translocation in HCO_3^--deficient medium, induces PLD1 translocation (Fig. 8) as well as PLD activation (Fig. 5). Because PLD was activated under these conditions, this translocation might indicate its activation. Thus, the data indicated that PKC is activated when PKA activity is blocked, and activation of PKA reduced PKC activity. Moreover, activation of PLD by dbcAMP is not inhibited by H-89 (Fig. 5), indicating that dbcAMP can activate PLD independently of PKA activity; however, there is no actin polymerization under these conditions (Fig. 6). We show in a recent work that actin polymerization in sperm depends upon PKA-dependent protein tyrosine phosphorylation (Brener et al., 2003). We also found that dbcAMP-dependent actin polymerization is completely blocked by the tyrosine kinase inhibitor genistein (not shown). This explains why the PKA inhibitor H-89 blocks dbcAMP-dependent actin polymerization.

To summarize this point, we suggest that PLD can be activated by PKC or by PKA pathways, leading to actin polymerization. In addition, PLD can be activated by cAMP independent of PKA, but this activation does not lead to actin polymerization.

To explain how inhibition of PKA activates PKC, we assumed that PKA inhibits PLC to form the PKC activator, diacylglycerol (DAG). Liu and Simon (1996) have shown that PKA specifically inhibits PLCβ2 isoform in COS-7 cells. This limits the activation of PKC due to the inhibition of DAG production. In this study, we showed that PKC-dependent actin polymerization induced by inhibiting PKA is completely blocked by the PLC inhibitor, U73122, whereas no inhibition is observed when PKC is activated by PMA, indicating the specificity of U73122 for PLC inhibition (Fig. 10). Moreover, we showed that PMA can induce PKC activation even in medium containing HCO_3^- when PKA is active, because there is no need for PLC activity in the presence of PMA (Fig. 7). Thus, the data suggest that PLC is activated when PKA is inhibited. This suggests that PKA inhibits PLC probably through its phosphorylation, resulting in PKC inhibition. This might also explain why activation of PKA stimulates PLD-dependent actin polymerization during capacitation. When PLC is blocked by PKA, the amount of available PIP_2 as a cofactor for PLD might be higher, resulting in PLD activation. This suggestion is supported by our finding in which PLD-dependent actin polymerization is blocked by inhibiting phosphatidyl inositol-4-kinase activity (our unpublished data), indicating that the cellular levels of PIP_2 controls PLD activity.

We previously described the gradual binding and activation of PLC in bovine sperm plasma membranes during capacitation (Spungin et al., 1995). This activation might cause significant PKC activation towards the end of the capacitation before the acrosome reaction. We also show that PKC is involved in bovine sperm acrosome reaction (Breitbart et al., 1992) probably in the activation of Ca^{2+} channels of the plasma membrane (Spungin and Breitbart, 1996). Here we showed that activation of PKC during capacitation caused relatively fast increase in F-actin formation which is followed by rapid F-actin breakdown. This F-actin breakdown probably occurred due to the high increase in $[Ca^{2+}]_i$ as was shown by us previously (Brener et al., 2003). Because an increase in F-actin formation during 4 h of incubation is necessary to achieve capacitation (Brener et al., 2003) and these conditions cannot be reached when PKC is highly activated (see Fig. 9), capacitation cannot be obtained. Thus, PKC activity should be kept low during sperm capacitation and this is accomplished by activation of PKA which clocks PLC or PKC activities. To enable PLC activation before the AR, the PKA activity should be downregulated towards the end of the capacita-

tion. Thus, it is possible that AC or PKA is partially inactivated towards the end of the capacitation conditions which allow PLC activation leading to PKC activation and achieving the AR. This possibility is supported by others who show inhibition of AC activity in capacitated sperm by adenosine via activation of A_1 receptor (Adeoya-Osiguwa and Fraser, 2002; Fraser and Adeoya-Osiguwa, 1999; Lefievre et al., 2002).

Thus, under nonphysiological conditions, activation of PKA or PKC can independently cause PLD-dependent actin polymerization. However, under physiological conditions, the PKA pathway is obligatory for actin polymerization leading to sperm capacitation, whereas the PKC pathway is mainly involved in acrosome reaction.

In summary, our results demonstrate that PKC and PKA can lead to actin polymerization, and a refined balance between the two pathways is required for optimal and sustained activation during sperm capacitation. The activation of PKA leads to inhibition of PLC and prevents PKC activation during capacitation. It appears that PKA activation would promote sperm capacitation whereas early activation of PKC during capacitation would jeopardize this process. Possible inhibition of PKA towards the end of the capacitation would cause activation of PKC to complete the acrosome reaction; however, this point should be clarified in the future.

Acknowledgments

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