

## ROLE OF PHOSPHOLIPASES DURING SPERM ACROSOMAL EXOCYTOSIS

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### 1. ABSTRACT

During fertilization the spermatozoon undergoes a process of regulated exocytosis triggered by oocyte-associated agonists. An early response generated by agonist-receptor interaction in spermatozoa is the activation of mechanisms leading to Ca<sup>2+</sup> influx, this ion being essential for the activation of phospholipases and for the fusion of the plasma membrane with the outer acrosomal membrane. Both a phosphoinositide-specific, and a phosphatidylcholine-specific phospholipase C are involved in the generation of a variety of diacylglycerol molecular species. Phospholipase D, on the other hand, does not seem to contribute (together with a phosphatidate phosphohydrolase) to a significant generation of diacylglycerol. Phospholipase A<sub>2</sub> action serves to release fatty acids and to generate lysophospholipids that, either directly or serving as substrates for the generation of other metabolites, have an essential role in the final stages of membrane fusion. Additional work needs to be carried out in the future to characterize which phospholipase isoenzymes are present in mammalian spermatozoa and mechanisms regulating these enzymes.

### 2. INTRODUCTION

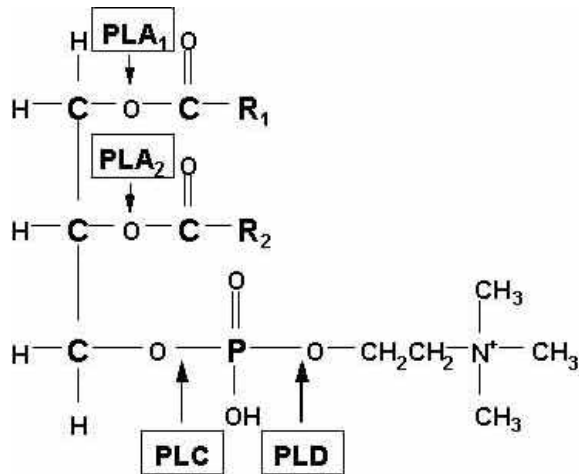
At the time of fertilization, the gametes must activate one another (1). The spermatozoon is activated by oocyte-associated agonists and undergoes a process of regulated exocytosis, the "acrosome reaction", which results in the release or exposure of enzymes contained in the acrosomal granule. Completion of exocytosis allows the fertilizing spermatozoon to penetrate oocyte vestment(s) and to fuse with the oolema (2) thus resulting in oocyte activation. In mammals, this activation is preceded by a necessary period of residence in the female tract during which a series of ill-defined changes collectively known as 'capacitation' takes place (2). There are a variety of agonists capable of initiating exocytosis in mammalian spermatozoa. It is thought that the main

agonists of exocytosis are the glycoprotein zona pellucida 3 (ZP3) (3,4) and progesterone (5). Both agonists interact during initiation of exocytosis, with progesterone priming the sperm cell to respond to ZP3 action (6). Other agonists may also have a role in the initiation of acrosomal exocytosis. Some agonists may, indeed, act as modulators or co-factors, as happens in other animal taxa. However, recent reports have indicated that many agonists are capable of initiating exocytosis in mammalian sperm cells and therefore it is difficult to understand which of these have physiological relevant functions. Among the recently identified agonists, the following can be mentioned: epidermal growth factor (EGF) (7-9), atrial natriuretic peptide (ANP) (10,11, but see 12,13, who provided evidence for a role of ANP in motility), prolactin (14), interleukin 6 (15), and *c-kit* (Stem Cell Factor) (16,17). gamma-Aminobutyric acid (GABA) can also be included in this list since it has been found that it triggers acrosomal exocytosis (6,18,19) and is present in oviductal fluid (20). However, it is also possible that GABA effects are just related to activation of receptors usually targeted by progesterone under physiological conditions. This, therefore, means that GABA should be considered as a probe rather than as a physiological agonist.

One of the very early responses generated upon agonist-receptor interaction in spermatozoa is the activation of mechanisms leading to Ca<sup>2+</sup> influx. Ca<sup>2+</sup> is essential for acrosomal exocytosis (2) since it is necessary for the activation of intracellular enzymes and for the actual fusion of membranes. Various Ca<sup>2+</sup>-dependent steps have been identified in the sequence underlying acrosomal exocytosis (21).

This review will concentrate on the activation of sperm phospholipases (figure 1) that play important roles in the generation of lipid messengers during acrosomal exocytosis. First, activation of phospholipase C (PLC) and their role in the generation of diacylglycerol (DAG) will

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**Figure 1.** Hydrolysis of phosphatidylcholine by phospholipases A<sub>1</sub>, A<sub>2</sub>, C and D. R<sub>1</sub> and R<sub>2</sub> are long-chain fatty acids, usually saturated in R<sub>1</sub> and unsaturated in R<sub>2</sub>.

be addressed. Second, the activation of phospholipase D (PLD) and its possible role during exocytosis will be discussed. Third, the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and the relevance for exocytosis of metabolites generated upon its activation will be summarised.

### 3. PHOSPHOLIPASE C

Stimulation of capacitated spermatozoa with the natural agonists progesterone or zona pellucida (ZP) causes a rise in DAG. This has been observed in capacitated mouse spermatozoa treated with progesterone or ZP (6), and in human spermatozoa stimulated with progesterone (22). Treatment with the Ca<sup>2+</sup> ionophore A23187 also leads to generation of DAG in a variety of species (ram: 23; mouse: 6; man: 22; boar: 24). This suggests that generation of DAG takes place after Ca<sup>2+</sup> entry when sperm cells are stimulated under physiological conditions. In fact, capacitated mouse or human spermatozoa stimulated with natural agonists do not show an elevation of DAG if Ca<sup>2+</sup> entry is inhibited by inclusion of Ca<sup>2+</sup> channel blockers (6,25).

DAG is an important metabolite during acrosomal exocytosis. It has become clear from various studies that DAG has a central role as lipid second messenger. DAG is now known to activate sperm PKC (26), and PLA<sub>2</sub> (27), and also to have a positive feedback effect on the PC-specific PLC (28).

There are three usual pathways generating DAG in cells: (a) hydrolysis of polyphosphoinositides (PPIs) by a specific PLC, (b) hydrolysis of phospholipids other than the PPIs by another type of PLC, and (c) hydrolysis of phospholipids such as phosphatidylcholine (PC) by PLD, which results in generation of phosphatidic acid (PA), followed by conversion of PA to DAG by phosphatidic acid phosphohydrolase (PPH).

#### 3.1. PPI-specific PLC (PIC)

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and phosphatidylinositol 4-phosphate (PIP) by a PPI-specific PLC, also known as phosphoinositidase C (PIC), has been demonstrated after

treatment with A23187 in spermatozoa of a variety of species (29). Similarly, hydrolysis of these phosphoinositides has been demonstrated in mouse (6) and human (30) spermatozoa after stimulation with physiological agonists of exocytosis.

It is not well known which types of PIC are involved in generation of DAG in spermatozoa in response to stimulation with natural agonists (reviewed in 31). Evidence for PIC-gamma activation, which is modulated by tyrosine phosphorylation, has been presented for mouse sperm (17,32). On the other hand, there is no direct evidence of activation of PIC-beta (which is activated by a pertussis toxin-insensitive GTP-binding proteins G<sub>q</sub> and G<sub>11</sub>) upon stimulation of spermatozoa, but both PIC-beta-1 and G-alpha-q/11 have been identified by Western blotting and localized to the acrosomal region of mouse spermatozoa (33). Its possible participation in acrosomal exocytosis has been inferred from the observation that progesterone-stimulated DAG formation is not blocked by pertussis toxin (34). The presence of other types of PICs has also been inferred. Spermatozoa may have a PIC activated by a pertussis toxin-sensitive GTP-binding protein (G<sub>o</sub> or G<sub>i</sub> type) since DAG formation stimulated by zona pellucida was blocked by pertussis toxin (34) and, furthermore, activation of G<sub>i</sub> proteins has been demonstrated after ZP stimulation of mouse spermatozoa (35). There could be yet another isoform of PIC which is activated by an elevation of intracellular Ca<sup>2+</sup> (PIC-delta ?) (see 31).

Hydrolysis of PIP and PIP<sub>2</sub> by PIC requires Ca<sup>2+</sup> in the extracellular medium when cells are stimulated with A23187 (29). Hydrolysis of PPIs does not take place if stimulation with A23187 is carried out in medium with EGTA (which chelates Ca<sup>2+</sup>), and no other bivalent cation tested (Mg<sup>2+</sup>, Sr<sup>2+</sup>, Ba<sup>2+</sup>) can replace for Ca<sup>2+</sup> (29). Activation of PIC and hydrolysis of PPIs in response to progesterone or ZP also requires elevation of intracellular Ca<sup>2+</sup> and this is due to entry of Ca<sup>2+</sup> from the extracellular space. As seen when quantifying DAG (as an indicator of PPI hydrolysis), DAG formation did not take place if Ca<sup>2+</sup> entry was prevented by inclusion of a series of Ca<sup>2+</sup> channel blockers (6,25). Thus, it seems that Ca<sup>2+</sup> entry precedes activation of PIC and formation of DAG.

In this context it is important to consider the other messenger generated by PIC action on PIP<sub>2</sub>, inositol 1,4,5-trisphosphate (IP<sub>3</sub>). For some time it was believed that this messenger had no relevant role in signalling during acrosomal exocytosis (36). This was due to the fact that, whereas in somatic cells generation of IP<sub>3</sub> precedes Ca<sup>2+</sup> entry and serves to elevate intracellular Ca<sup>2+</sup> by release of this cation from stores located in the endoplasmic reticulum, no parallel situation exists in spermatozoa. As indicated above, activation of sperm PIC, and hence generation of DAG and IP<sub>3</sub> takes place after Ca<sup>2+</sup> entry and, moreover, spermatozoa do not have an endoplasmic reticulum. However, recent studies have revealed that the acrosome may serve functions similar to those played by the endoplasmic reticulum in somatic cells. Experiments using thapsigargin, an inhibitor of endoplasmic reticulum Ca<sup>2+</sup>-ATPase, revealed release of Ca<sup>2+</sup> from a putative acrosomal 'store' (33,37). Also, a receptor for IP<sub>3</sub> has been localized to the acrosomal granule in a variety of species, and it has been found that IP<sub>3</sub> triggers Ca<sup>2+</sup> release in

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permeabilized spermatozoa (33). It is thus possible that although activation of PIC, hydrolysis of PPIs, and generation of DAG and IP<sub>3</sub> require elevation of intracellular Ca<sup>2+</sup> due to influx, the IP<sub>3</sub> generated could target an IP<sub>3</sub> receptor on the outer acrosomal membrane to release additional Ca<sup>2+</sup>. This will be consistent with the finding that early events underlying acrosomal exocytosis require low (micromolar) concentrations of Ca<sup>2+</sup> whereas late events in the sequence, including membrane fusion itself, require higher (millimolar) levels of the cation (21,29).

A PLC from human sperm specific for the PPIs has been partially purified and characterized (38). It was found to hydrolyze phosphatidylinositol, PIP and PIP<sub>2</sub> and to be maximally activated in the presence of 5 mM Ca<sup>2+</sup>. However, one half of maximal activity was still observed without any Ca<sup>2+</sup> addition indicating that endogenous Ca<sup>2+</sup> was sufficient to support a significant proportion of activity. Inclusion of EGTA almost totally inhibited the enzyme's activity. These results agree well with observations of Ca<sup>2+</sup> requirements in live spermatozoa stimulated with A23187 in the presence of different concentrations of Ca<sup>2+</sup> or EGTA (29). Analysis of subcellular fractions revealed that around 55% of the enzyme activity appeared in a soluble form, and after separation of sperm heads and tails, it was found that 80% of particulate PLC activity was located in the sperm head (38). A PPI-specific PLC was also purified from bull sperm (39) but, when sperm heads and tails were separated and the enzyme activity analysed after homogenization, the tail fraction contained higher specific activity.

Several studies have demonstrated that activation of PIC and hydrolysis of PPIs are essential for acrosomal exocytosis. In ram spermatozoa stimulated with A23187, inclusion of neomycin (an antibiotic that binds to PPIs and prevents their hydrolysis) results in no exocytosis (29). Inhibition by neomycin is overcome by inclusion of DAG (one of the products of PIC action on PPIs), thus demonstrating the specificity of the effect (23). Moreover, generation of DAG in human spermatozoa in response to progesterone was blocked by inclusion of either neomycin or the PIC inhibitor U73122, with the inactive structurally-related compound U73343 having no effect (22). In parallel experiments, exocytosis triggered by progesterone was blocked by neomycin or U73122, again with U73343 having no effect.

The importance of PIC-mediated generation of DAG relates to the fact that this metabolite has several functions as messenger. In any case, it has also been examined whether DAG acts as a substrate for the generation of other active metabolites such as PA or MAG. It seems that neither DAG conversion to PA via DAG kinase, nor DAG catabolism to MAG via DAG lipase are key events. Inhibition of catabolism of DAG via DAG kinase enhanced exocytosis which suggests that DAG rather than PA is the important metabolite (22,23). Similarly, inhibition of DAG lipase also led to enhancement of exocytosis, again indicating that DAG and not a derived metabolite is important in signalling (23).

### 3.2. PC-specific PLC

It has been argued that in somatic cells the main route for DAG formation from phospholipids other than the phosphoinositides involves the concerted action of PLD

and PPH (40,41) and the same has been assumed for mammalian spermatozoa (42). However, the possible role of a PC-specific PLC (PC-PLC) has also been recognized in somatic cells (43,44) and a series of studies has shown that, in fact, this may be the major route for DAG generation in mammalian spermatozoa.

Spermatozoa stimulated with A23187, progesterone or ZP experienced hydrolysis of diacyl- or alkylacyl-PC, with a concomitant rise in DAG or alkylacylglycerol and no or very little subsequent changes in PA or alkyl-PA, indicative of PLC activity (6,22,28). This agrees well with an earlier study showing that mammalian sperm have a PC-specific PLC localized to the acrosomal region (45). This PC-PLC has been purified and shown to generate DAG from [1-<sup>14</sup>C]dioleoyl-PC, but not from similarly labelled phosphatidylinositol or phosphatidylethanolamine (45); this is coincident with the source of DAG identified in a study in which sperm phospholipids were labelled with radioactive precursors (28). Thus, it appears that in mammalian spermatozoa DAGs are being generated directly by PLC-mediated hydrolysis of PC.

Other studies have also detected PLC activity, not related to phosphoinositides, in rabbit, boar, bull, and mouse spermatozoa (46-49). However, some of these results should be evaluated critically because conditions used for assays not always allowed discrimination of PC-specific activity, or PLC- from PLD-mediated hydrolysis. For instance, use of substrates with labelled choline and quantification of radioactivity in the whole upper aqueous phase after lipid extraction does not discriminate between choline (the product of PLD) and choline-phosphate (the product of PLC) (49).

Very little PA seems to be formed from the DAG that is generated by PC-PLC (6,22,28). The reason for this may be that the fatty acid composition of PC-derived DAG is different from the PPI-derived DAG and that DAG kinase has a substrate preference for DAG deriving from phosphoinositides.

## 4. PHOSPHOLIPASE D

There is clear evidence that PLD plays an important role in signalling during the acrosome reaction of sea urchin spermatozoa (50). In fact, it seems that most of the DAG generated upon stimulation with a fucose-sulfate glycoconjugate is obtained via hydrolysis of PC by PLD with the resulting PA being converted to DAG by PPH. In mammals, however, all the studies carried out so far to test the participation of PLD in events underlying acrosomal exocytosis suggest that this phospholipase does not really contribute to generate DAG and the little PLD activity that is detected appears to have little importance in the sequence of signalling events culminating in membrane fusion.

Studies on ram, mouse, boar, and human spermatozoa, stimulated with A23187, progesterone, or ZP, revealed no or very little generation of PA upon stimulation and in no case was PA generated before DAG (6,22,24,51). PLD activity was also examined using the ability of this enzyme to catalyze a transphosphatidylation reaction in the presence of ethanol (a reaction that unambiguously identifies PLD activity; 41) and which

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results in the formation of phosphatidylethanol at the expense of PA. No or very little formation of phosphatidylethanol was observed in spermatozoa from ram, mouse or man upon stimulation (6,22,51). Changes of diglycerides when sperm cells were stimulated in the presence of ethanol have also been studied; the rationale behind this experimental approach is that since less PA would be generated by PLD when ethanol is present, then less diglycerides should be formed upon stimulation. Quantification of DAG or alkyl-acyl-glycerol generation revealed no decrease after stimulation in the presence of ethanol as compared to spermatozoa stimulated in the absence of ethanol (51). Another way of exploring the PLD-PPH pathway involvement in signalling is by using the PPH inhibitor propanolol. No decrease in DAG formation was noticed after stimulation in the presence of this compound (51). The relevance of these findings for exocytosis relates to the fact that in parallel experiments stimulation of spermatozoa in the presence of ethanol or propanolol did not affect occurrence of acrosome reactions (51).

## 5. PHOSPHOLIPASE A<sub>2</sub>

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) acts on membrane phospholipids releasing the fatty acid in position 2 of the glycerol backbone and thus generating a lysophospholipid (figure 1). The action of PLA<sub>2</sub> on PC would generate lysoPC and fatty acids such as arachidonic acid. It has to be borne in mind that the action of PLA<sub>2</sub> on other phospholipids such as phosphatidylethanolamine (PE) or phosphatidylinositol (PI) would yield lysoPE or lysoPI, respectively, and that these lysophospholipids may also be important metabolites (52). Furthermore, arachidonic acid is not the only fatty acid in PC, PE or PI. Other unsaturated fatty acids (such as oleic, linoleic or linolenic acid, or docosahexaenoic acid) are also present in position 2 of these phospholipids (53) and, upon release by the action of PLA<sub>2</sub>, could also have a biological function. In addition, phospholipids in sperm membranes are not only diacyl lipids; they could also be alkyl-acyl-phospholipids (53) and in this case the action of PLA<sub>2</sub> would generate and alkyl-lysophospholipid.

### 5.1. Types of PLA<sub>2</sub> in spermatozoa

PLA<sub>2</sub> constitutes a large superfamily of enzymes (54,55) and several PLA<sub>2</sub> groups (I through IX) have been established based on sequence information. PLA<sub>2</sub>s are either secreted, low molecular weight enzymes (13-18 kDa) which, in general terms, require millimolar levels of Ca<sup>2+</sup>, or they are cytosolic enzymes of larger size (two groups of 85 kDa forms, and one of 29 kDa) requiring either no Ca<sup>2+</sup> or micromolar levels of the cation (55; and references therein).

There is, unfortunately, little information on which are the PLA<sub>2</sub> isoenzymes present in mammalian spermatozoa. Efforts have been directed towards a biochemical characterisation of PLA<sub>2</sub> in sperm from various species and information agrees on the fact that sperm PLA<sub>2</sub> requires millimolar levels of Ca<sup>2+</sup> (56; and see below). It should nevertheless be noted that most studies have used protocols of acid extraction of PLA<sub>2</sub> and, thus, that any possibility of detecting high molecular weight cytosolic isoenzymes has been lost with this procedure (57).

Partial purification and characterization has revealed the existence of a sperm PLA<sub>2</sub> of about 14-16 kDa. Unfortunately there is only limited sequence data, and it is thus difficult to assign the sperm PLA<sub>2</sub> to any of the groups recognized by Dennis (55). In any case, partial sequencing of the N-terminal region of a human sperm PLA<sub>2</sub> has shown some similarities with secretory PLA<sub>2</sub>s of groups I and II (from snake venom, and porcine/human pancreas), although this human sperm PLA<sub>2</sub> appears to represent a novel sequence (58). Interestingly, antibodies raised against cobra (*Naja naja*) venom recognize a 16 kDa protein in SDS-extracts from bull spermatozoa (59). Similarly, antibodies against porcine pancreas PLA<sub>2</sub> recognize a protein in hamster and human sperm (60,61). The antibodies have also been used in immunolocalization work; PLA<sub>2</sub> has been detected in the acrosomal region, as well as in other sperm compartments. The physiological relevance of these findings is suggested by the fact that Fab fragments of the antibody against porcine pancreas PLA<sub>2</sub> are capable of inhibiting acrosomal exocytosis in hamster spermatozoa (62).

Recent work has identified expression of a group IIC PLA<sub>2</sub> in various spermatogenic cells in the mouse; this PLA<sub>2</sub> is expressed only in testis. Expression of the *Pla2g2c* gene has been detected in mouse pachytene spermatocytes, secondary spermatocytes and round spermatids (63). It is thus possible that this PLA<sub>2</sub> is present in mature mouse spermatozoa but differences may exist between species because in humans, *PLA2G2C* appears to be a nonfunctional pseudogene (64).

In somatic cells, a cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) of larger size (85 kDa) has been found to be activated during exocytosis. There is no indication as to whether this isoenzyme is present in mammalian sperm. In any case, recent experiments in which the gene coding for this cPLA<sub>2</sub> has been mutated showed that homozygous male mice with the mutations had no impairment in their fertility (65,66), suggesting that this cPLA<sub>2</sub> may not be essential in events underlying sperm function, including exocytosis.

Studies on PLA<sub>2</sub> in reproductive tissues have identified and characterized PLA<sub>2</sub> activities in bull prostate, seminal vesicle, Cowper's gland and in seminal plasma (67,68) and in human seminal plasma (69). Antibodies generated against PLA<sub>2</sub> purified from bovine seminal vesicle fluid blocked PLA<sub>2</sub> activity in bovine seminal plasma, seminal vesicles and its fluid, and gave single precipitation lines with the same samples, but no cross-reactivity was detected with other reproductive tissues or human seminal plasma (70). Using this antibody, PLA<sub>2</sub> was localized in the apical part of epithelia in bull seminal vesicle and immunoreactivity was also seen in ejaculated bull spermatozoa (restricted to acrosomal and post-acrosomal region, and the midpiece) but it was not present on the surface of epididymal spermatozoa (70). These results suggest that different PLA<sub>2</sub>s may be present in seminal plasma, and that PLA<sub>2</sub>s may become bound to the surface of spermatozoa upon ejaculation. This may not be true for other species such as mouse, where surface-active PLA<sub>2</sub> was found in epididymal spermatozoa (71).

The PLA<sub>2</sub> identified in bull reproductive tissues has been purified and characterized (67). The PLA<sub>2</sub> activity detected in human seminal plasma, which reacted with

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monoclonal antibodies raised against human synovial fluid PLA<sub>2</sub> (69), has also been purified and the amino acid sequence of the N-terminal 20 residues was found to be identical to that of human synovial PLA<sub>2</sub>. Recently, a novel PLA<sub>2</sub> from bull seminal plasma has also been purified (72) and found to be a 60 kDa enzyme. In fact, it has now been shown to be a platelet-activating-factor acetylhydrolase, although it is capable of hydrolysing long-chained phosphatidylcholine (73). Since these enzymes can become bound to the surface of spermatozoa it would be interesting to test how much of the PLA<sub>2</sub> activity detected in spermatozoa relates to PLA<sub>2</sub>s of seminal plasma that are bound to the sperm surface.

### 5.2. Sperm PLA<sub>2</sub> - Biochemical data

Probably the majority of the studies carried out so far have analyzed (although not deliberately) the activity of the low molecular weight PLA<sub>2</sub>, since methods used for extraction of enzyme activity involved acid treatment and this leads to inactivation of the high molecular weight cytosolic isoform (57).

Studies on sperm PLA<sub>2</sub> have shown that in all mammalian species examined so far the activity of this enzyme is Ca<sup>2+</sup>-dependent. Sonicates of human sperm (ejaculated) have the highest specific activity (805.4 nmol / h / mg protein) followed by mouse (epididymal), rabbit (epididymal), ram (ejaculated) and bull (ejaculated) whose activities are 62.1, 6.0, 0.7 and 0.2 nmol / h / mg protein, respectively (56,71,74).

PLA<sub>2</sub> from ejaculated bull spermatozoa assayed *in vitro* can hydrolyse linoleic- and arachidonic-PE, as well as linolenic- and arachidonic-PC (and also a fluorescent derivative of PC) (67). Ram sperm PLA<sub>2</sub> assayed *in vitro* hydrolyses both PE and PC but it appears to prefer PE as substrate over PC (75). In intact ram sperm labelled with radioactive arachidonic acid, the source of most free arachidonic acid upon stimulation with A23187 was PC, with less contributions from PE and PS. The latter is probably due to the fact that less PE actually exists in sperm membranes (53).

It has been found that sperm PLA<sub>2</sub> activity can be inhibited by compounds known to affect PLA<sub>2</sub> from other cellular systems. However, PLA<sub>2</sub> from spermatozoa of different species vary in their sensitivity to inhibitory compounds. For example, mepacrine (=quinacrine) blocks golden hamster, guinea pig and mouse sperm PLA<sub>2</sub> activity (71,74,76,77) but does not affect ram (56) or human sperm PLA<sub>2</sub> (74, but see 78). On the other hand, p-bromophenacyl bromide (pBPB) inhibits the activity of PLA<sub>2</sub> from all the species examined so far: golden hamster, guinea pig, mouse and man (71,74,76-78). Finally, dexamethasone, although capable of inhibiting PLA<sub>2</sub> from various cells (79,80) including ram spermatozoa (56), does not inhibit mouse sperm PLA<sub>2</sub> (71). The reasons why sperm PLA<sub>2</sub> differ in sensitivity to classic, though probably less specific inhibitors remain obscure. We have examined the sensitivity of ram sperm PLA<sub>2</sub> to recently developed, more specific compounds such as chloracysine and the two alkylamines Ro-4493 and Ro-4639 (56) both in *in vitro* assays and in labelled, stimulated cells. Enzyme activity in sperm sonicates is completely abolished by these reagents in a fashion similar to their effects on PLA<sub>2</sub>

from porcine pancreas or human neutrophil cytoplasm (81,82). *In vivo*, most of these reagents had a deleterious effect on sperm viability. The compound Ro-4493, however, was a useful tool to demonstrate a link between arachidonic acid release and occurrence of exocytosis (52).

### 5.3. Activation of PLA<sub>2</sub> during acrosomal exocytosis

For a long time, various studies have addressed whether PLA<sub>2</sub> was involved in sperm acrosomal exocytosis. However, most studies have contributed circumstantial evidence: (a) Spermatozoa from several species (hamster, guinea pig, man) treated with putative PLA<sub>2</sub> inhibitors (mepacrine or pBPB) failed to undergo an acrosome reaction either spontaneously or in response to ionophore treatment (77,83-85); and (b) spermatozoa treated with exogenous metabolites generated by PLA<sub>2</sub> (i.e. lysophospholipids or fatty acids) experienced a stimulation/acceleration of acrosomal exocytosis (86-88). None of these studies have actually quantified activity of PLA<sub>2</sub> (e.g. by labelling cells and measuring fatty acid release) and therefore do not provide direct evidence of PLA<sub>2</sub> participation in exocytosis. There has been a limited attempt at labelling human sperm with radioactive arachidonic acid, and then follow changes upon treatment with A23187 (89). A rise in free arachidonic acid, paralleled by decreases in PC and PI were observed after stimulation, but no clear evidence of coupling between these changes and the occurrence of exocytosis was provided.

Recent studies have supplied thorough evidence demonstrating that PLA<sub>2</sub> plays an essential role in the release of fatty acids and lysophospholipids involved in sperm membrane fusion during acrosomal exocytosis (52). Using ram spermatozoa as model species, it was possible to label sperm phospholipids using radioactive arachidonic acid as precursor. Stimulation of Ca<sup>2+</sup> entry with the ionophores A23187 or ionomycin revealed a time- and concentration-dependent increase in free fatty acids and, in cells labelled with [<sup>32</sup>P]P<sub>i</sub>, a parallel rise in lysoPC. This, together with the fact that no DAG-derived arachidonic acid release was observed, clearly indicates PLA<sub>2</sub> activation upon sperm stimulation. A tight coupling between arachidonic acid release and exocytosis was observed when the time-course of both phenomena were compared and also a good agreement was found between the concentration-dependence of A23187-stimulated arachidonic acid release and occurrence of exocytosis. Further evidence for an essential role of PLA<sub>2</sub> in exocytosis derives from the finding that the PLA<sub>2</sub> blocker Ro-4493 inhibited both arachidonic acid release and exocytosis, and that exocytosis in cells stimulated with A23187 in the presence of Ro-4493 was restored if either arachidonic acid or lysoPC were included. These results suggest very strongly that PLA<sub>2</sub> may have an essential role in the acrosome reaction initiated by natural agonists.

PLA<sub>2</sub> is activated in human (90) and boar spermatozoa (91) in response to progesterone but, to the best of our knowledge, no studies have yet reported ZP-induced activation of PLA<sub>2</sub>. Interestingly, reagents known to activate GTP-binding proteins, a transduction event involved in ZP-triggered acrosome reaction (34), trigger exocytosis and this is inhibited by PLA<sub>2</sub> blockers, which suggests a link between these two events (92).

### 5.4. Regulation of sperm PLA<sub>2</sub>

Extensive work has been done on the characterization of PLA<sub>2</sub> in many cells and it has been found that mechanisms of PLA<sub>2</sub> regulation differ between cell systems. Evidence has been gathered in favour of the following mechanisms of PLA<sub>2</sub> regulation: (a) G-protein mediated PLA<sub>2</sub> activation, (b) activation of the enzyme through a rise in intracellular Ca<sup>2+</sup>, (c) activation via phosphorylation by MAP kinase, and (d) modulation of PLA<sub>2</sub> activity by DAG. Although in some cells DAG may stimulate PKC which, in turn would phosphorylate MAP kinase and this, in turn, would activate PLA<sub>2</sub>, in some cells DAG appears to stimulate PLA<sub>2</sub> directly without the participation of the PKC pathway (93-96). It has been argued that since PKC and PLA<sub>2</sub> contain similar regulatory sequences (97), DAG may bind directly to PLA<sub>2</sub> and activate it, in a way similar to its mode of action on PKC (98). In spermatozoa little is still known about the mechanisms that regulate the activation of this enzyme, and the regulation of the different PLA<sub>2</sub> isoforms (if several are in fact present).

One possible mechanism regulating PLA<sub>2</sub> in spermatozoa is that involving G proteins (92). It is not clear whether G proteins are directly related to PLA<sub>2</sub> activity or, more likely, whether G proteins activate a signalling pathway such as Ca<sup>2+</sup> influx, or PIC mediated DAG generation, that eventually results in PLA<sub>2</sub> activation.

Another possible pathway modulating PLA<sub>2</sub> is the DAG-PKC-MAP kinase cascade (99). The direct activation of PLA<sub>2</sub> by PKC has been postulated (42) but, so far, there is no evidence for this interaction *in vivo* (100). On the other hand, MAP kinase can also be phosphorylated by the cAMP-PKA pathway (101) and this could be one additional mechanism modulating PLA<sub>2</sub> in sperm (102).

Evidence for PKC-mediated phosphorylation of a variety of substrates has been now demonstrated in human spermatozoa after stimulation with progesterone (26). In addition, components of the MAP kinase pathway, such as Ras (103), and ERK 1 and 2 (104), have been identified in spermatozoa. MAP kinase activation in response to A23187 or progesterone has also been demonstrated in human spermatozoa (104,105). However, it is not yet clear whether MAP kinase has any role in events underlying exocytosis in spermatozoa (105) and, furthermore, no evidence for the high molecular weight cytosolic PLA<sub>2</sub> (the PLA<sub>2</sub> activated by MAP kinase) has been reported. Interestingly, studies on mice in which the gene coding for the cPLA<sub>2</sub> has been mutated showed that homozygous male mice with the mutations had no impairment in their fertility (65,66) suggesting that the cPLA<sub>2</sub> may not have an essential role in acrosomal exocytosis (see discussion in 106).

Finally, sperm PLA<sub>2</sub> activity could be modulated directly by DAG, independently of PKC activation. Our results have shown that DAG is capable of enhancing the activity of ram sperm PLA<sub>2</sub> in an *in vitro* enzyme assay (56). In addition, alkyl-acyl-glycerols can also enhance the activity of the enzyme *in vitro* (107), suggesting that a variety of PC-derived diglycerides could participate in modulation of the enzyme. In intact labelled cells, the

addition of permeable DAGs resulted in an enhancement of PLA<sub>2</sub> activity; the significance of this observation is related to the fact that under similar conditions exocytosis was enhanced (27). Using an alternative approach, in which endogenous levels of DAG were kept high by the inclusion of the DAG kinase inhibitors R59022 or R59949 (22,23), an enhancement of both PLA<sub>2</sub> activity and exocytosis was also demonstrated (27). The effect of exogenous diglycerides was evident when both 1,2- and 1,3-DAGs, as well as alkyl-acyl-glycerol (27), were included. This indicates that the action of the diglycerides is not mediated by PKC, since neither 1,3-DAG nor alkyl-acyl-glycerol are capable of activating the kinase.

### 5.5. Roles of lysophospholipids and fatty acids

These metabolites seem to serve as co-activators of some types of protein kinase C (108,109), but their major role could relate to perturbation of cell membranes during fusion and this has been extensively investigated in mammalian spermatozoa (see 2).

The likelihood that lysophospholipids, such as lysoPC, play a role in acrosomal exocytosis has been indicated by some early studies in which pretreatment of spermatozoa with lysoPC shortened capacitation and resulted in a rapid induction of acrosomal exocytosis after addition of millimolar Ca<sup>2+</sup> (86). Additional studies have confirmed that lysoPC triggers the reaction in spermatozoa of the hamster (110,111), and bull (112). In spermatozoa in which exocytosis is triggered by the ionophore A23187, lysoPC exerts a concentration-dependent enhancement of exocytosis (52). Further evidence for its role in exocytosis comes from experiments in which PLA<sub>2</sub> activity was blocked (and hence, lysoPC and fatty acid release was inhibited) and inclusion of exogenous lysoPC restored the ability of cells to undergo an acrosome reaction (ram: 52; hamster: 111). It is interesting that not all lysophospholipids seem capable of exerting a stimulatory role on exocytosis. Thus, lyso PC and lysoPI were capable of enhancing acrosomal exocytosis, whereas lysophosphatidylserine (lysoPS) was not (52,111). In fact, lysoPS appears to have an inhibitory role under certain conditions (113).

Further to its direct role on membranes, one type of lysoPC (alkyl-lysoPC) may serve as substrate for the formation of an important lipid: alkyl-acetyl-PC (also known as "platelet activating factor" or PAF). Stimulation of human spermatozoa with A23187 or progesterone (90) or ram spermatozoa with A23287 (E.R.S. Roldan, unpublished results) causes formation of PAF. This lipid can stimulate acrosomal exocytosis (52,114) although it is not yet clear whether PAF acts on a membrane receptor in an autocrine fashion. Experiments in which ram spermatozoa were stimulated to undergo an acrosome reaction revealed that the PAF receptor inhibitor WEB 2086 did not affect the time-course of exocytosis (E.R.S. Roldan, unpublished results). On the other hand, PAF-induced acrosome reactions in human spermatozoa were blocked by the PAF receptor inhibitor L659989 (although not all sperm samples responded to PAF treatment) (115). PAF also induced tyrosine kinase activity in human spermatozoa although it did not cause an increase in intracellular Ca<sup>2+</sup> (see 114,115).



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