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Sperm phospholipases and acrosomal exocytosis

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1. ABSTRACT

At the time of fertilization, the sperm cell undergoes regulated exocytosis in response to the oocyte-associated agonists progesterone and zona pellucida. An early response generated by agonist-receptor interaction in spermatozoa is the activation of mechanisms leading to Ca^{2+} 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 play a significant role in the generation of diacylglycerol. Hydrolysis of phospholipids by phospholipase A_2 generates free fatty acids and lysophospholipids; these are important either as substrates for the generation of other metabolites (e.g., eicosanoids) or having a direct, essential action in the final stages of membrane fusion. There is still much work to be done in the future in order to characterize phospholipase isozymes and their regulation during acrosomal exocytosis in spermatozoa.

2. INTRODUCTION

During fertilization, the gametes interact and activate each other (1). The spermatozoon is stimulated by oocyte-associated agonists and undergoes a process of regulated exocytosis, the so-called “acrosome reaction”, which results in the release or exposure of enzymes contained in the acrosomal granule. Completion of exocytosis allows the fertilizing spermatozoon to penentrate the oocyte vestment(s) and to fuse with the oolema (Yanagimachi 1994; Kopf 2002) thus initiating the activation of the oocyte. In mammals, spermatozoa need a prior period of residence in the female tract during which a series of ill-defined changes, collectively known as "capacitation", takes place (2,3).

There are a variety of agonists capable of initiating exocytosis in mammalian spermatozoa but it is thought that the main agonists of exocytosis are the glycoprotein zona pellucida 3 (ZP3) (4,5) and progesterone (6). Both agonists interact during initiation of exocytosis, with progesterone priming the sperm cell to respond to ZP3 action (7,8). Other agonists that can trigger acrosomal

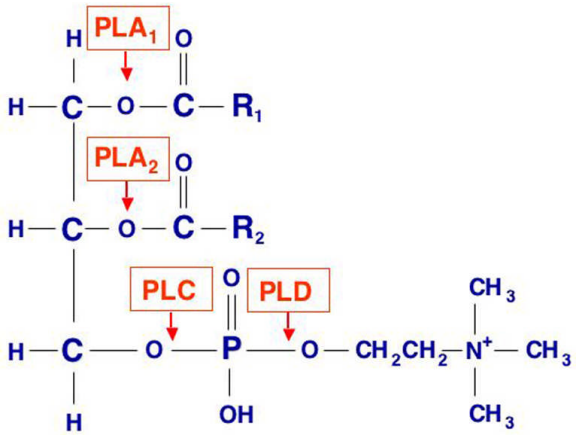

Figure 1. Hydrolysis of phosphatidylcholine by phospholipases A_1, A_2, C and D. R1 and R2 are long-chain fatty acids, usually saturated in R1 and unsaturated in R2.

exocytosis may, perhaps, act as modulators or co-factors, as happens in other animal taxa, but the physiological importance of several of them remains to be established. The following have been identified as capable of initiating acrosomal exocytosis: epidermal growth factor (EGF) (9-11), atrial natriuretic peptide (ANP) (12,13, but see 14,15, who provided evidence for a role of ANP in motility), prolactin (16), interleukin 6 (17), and *c-kit* (Stem Cell Factor) (18,19). gamma-Aminobutyric acid (GABA) is also known to initiate acrosomal exocytosis in various species (7,20-22) but it is not clear whether it has a physiological role *in vivo*. GABA is present in oviductal fluid (23) so it may be acting as a co-factor during initiation of acrosomal exocytosis but it is also possible that GABA effects are just related to activation of receptors usually targeted by progesterone under physological conditions.

One of the very early responses generated upon agonist-receptor interaction in spermatozoa is the activation of mechanisms leading to Ca^{2+} influx. Ca^{2+} is essential for acrosomal exocytosis (2,3) since it is necessary for the activation of intracellular enzymes and for the actual fusion of membranes. Various Ca^{2+}-dependent steps have been identified in the sequence underlying acrosomal exocytosis (24).

This review will concentrate on three groups of sperm phospholipases that play important roles in the generation of lipid messengers during acrosomal exocytosis (Figure 1): (a) phospholipases C (PLC) and their role in the generation of diacylglycerol (DAG), (b) phospholipase D (PLD) and its possible role during exocytosis, and (c) phospholipase A_2 (PLA_2) and the relevance for exocytosis of metabolites generated upon its activation. Other possible functions of sperm phospholipases will also be briefly presented.

3. PHOSPHOLIPASE C

Phospholipase C (PLC) actually represents a large group of hydrolases that generate 1,2-diacyglycerol (DAG). Two main groups can be distinguished, one comprising PLCs specific for phosphoinositides, and referred to as phosphoinositide-specific PLC (PPI-PLC) or phosphoinositidase C (PIC), and another one including PLCs specific for phosphatidylcholine (PC), known as PC-PLC. PPI-PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate generates 1,4,5-trisphosphate (IP_3) and DAG. On the other hand, PC-PLC hydrolysis of phosphatidylcholine results in choline phosphate and DAG. In many studies in somatic or germ cells, no distinction has been made between these two groups with the generic name PLC usually employed in connection to PPI-PLC. In the context of this review it will be important to distinguish them since each may have specific roles and different regulatory mechanisms.

Several studies have characterized the generation of DAG and its role during acrosomal exocytosis in spermatozoa. 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 (7), and in human spermatozoa stimulated with progesterone (25). Treatment with the Ca^{2+} ionophore A23187 also leads to generation of DAG in a variety of species (ram: 26; mouse: 7; man: 25; boar: 27). This suggests that generation of DAG takes place after Ca^{2+} 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^{2+} entry is inhibited by inclusion of Ca^{2+} channel blockers (7,28).

DAG is an important metabolite during acrosomal exocytosis because it has a central role as lipid second messenger. DAG is now known to activate sperm protein kinase C (PKC)(29), and phospholipase A_2 (PLA_2)(30), and also to have a positive feedback effect on the PC-specific PLC (31). In addition to its role as messenger, DAG may serve as substrate for the generation of other active metabolites such as phosphatidic acid (PA) or monoacylglycerol (MAG). However, it seems that neither DAG conversion to PA via DAG kinase, nor DAG catabolism to MAG via DAG lipase are important signalling events in spermatozoa. Inhibition of catabolism of DAG via DAG kinase enhanced exocytosis which suggests that DAG rather than PA is the important metabolite (25,26). Similarly, inhibition of DAG lipase also led to enhancement of exocytosis, again indicating that DAG, and not a derived metabolite, is important in signalling (26).

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

3.1. Phosphoinositide-specific PLC (PIC)

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) by a phosphoinositide-specific PLC has been demonstrated after treatment with A23187 in spermatozoa of a variety of mammalian species (32). Similarly, hydrolysis of these phosphoinositides has been demonstrated in human spermatozoa after stimulation with progesterone (33), in mouse spermatozoa after stimulation with progesterone or ZP (7) and in guinea pig spermatozoa stimulated with GABA or progesterone (34). In agreement with the proposed priming role of progesterone, mouse spermatozoa stimulated with the steroid and ZP exhibited significantly higher hydrolysis of both PIP_2 and PIP (7).

Hydrolysis of PIP and PIP_2 by PIC requires Ca^{2+} in the extracellular medium when cells are stimulated with A23187 (32). Hydrolysis of PPIs does not take place if stimulation with A23187 is carried out in medium with EGTA (which chelates Ca^{2+}), and no other bivalent cation tested (Mg^{2+}, Sr^{2+}, Ba^{2+}) can replace for Ca^{2+} (32). Activation of PIC and hydrolysis of PPIs in response to progesterone or ZP also requires elevation of intracellular Ca^{2+} and this is due to entry of Ca^{2+} from the extracellular space. As seen when quantifying DAG (as an indicator of PPI hydrolysis), DAG formation after progesterone treatment did not take place if Ca^{2+} entry was prevented by inclusion of a series of Ca^{2+} channel blockers (7,28). Thus, Ca^{2+} entry may precede activation of PIC and formation of DAG and IP_3. If Ca^{2+} entry precedes PIC activation, then the question that arises is how is Ca^{2+} entry regulated in the first place since it is generally believed that intracellular Ca^{2+} rises are regulated by IP_3-mediated actions. In addition, it is important to consider whether the IP_3 generated by PIC has any role in intracellular Ca^{2+} regulation in spermatozoa.

In many cells, PIC activation does not require Ca^{2+} entry and one of the early cell responses is intracellular Ca^{2+} elevation, by release from stores, which is mediated by PIC-generated IP_3. For some time it was believed that IP_3 had no relevant role in signalling during acrosomal exocytosis (35) because, as mentioned, PIC activation required intracellular Ca^{2+} elevation, IP_3 formation was triggered by Ca^{2+} entry, inositol 1,3,4,5-tetrakisphosphate (IP_4), a derivative of IP_3 that releases Ca^{2+} from stores, was not detected in spermatozoa, and Ca^{2+} stores were thought to be absent from spermatozoa due to their lack of endoplasmic reticulum.

However, several lines of evidence currently suggest that the acrosome may serve functions similar to those played by the endoplasmic reticulum in somatic cells: (a) Ca^{2+} has been shown to selectively accumulate in the acrosome, as revealed by various Ca^{2+}-sensitive fluorescent probes (36), (b) thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, induced a release of Ca^{2+} from a putative acrosomal store which was followed by acrosomal exocytosis (36-38), (c) calreticulin, the major Ca^{2+}-binding/store protein in the endoplasmic reticulum has been localized to the acrosome of rat and human spermatozoa (39,40), (d) IP_3 receptors have been immunolocalized to the acrosomal granule in a variety of species (37,40), (e) IP_3 triggers Ca^{2+} release in permeabilized spermatozoa (37), and (f) thimerosal, an IP_3 receptor agonist, induces acrosomal exocytosis (36).

It is thus possible that although activation of PIC (leading to hydrolysis of PPIs, and generation of DAG and IP3) requires elevation of intracellular Ca^{2+} due to influx, the IP_3 generated could target an IP_3 receptor on the outer acrosomal membrane to release additional Ca^{2+} from stores. Emptying of stores could, in turn, set in train capacitative Ca^{2+} entry (i.e. opening of store-operated channels) (41,42) for a massive elevation of intracellular Ca^{2+} levels. This will be consistent with the finding that early events underlying acrosomal exocytosis require low (micromolar) concentrations of Ca^{2+} whereas late events in the sequence, including membrane fusion itself, require high (millimolar) levels of the cation (24,32).

These ideas are in agreement with studies and hypotheses of mechanisms underlying the two phases of intracellular Ca^{2+} increase seen after initiation of acrosomal exocytosis in response to ZP stimulation (43,44): The early events of ZP signal transduction in spermatozoa include opening of T-type, low voltage-activated Ca^{2+} channels (45,46), resulting in a transient Ca^{2+} influx and the activation of the heterotrimeric G proteins G_{i1} and G_{i2} (65). These initial responses produce an activation of PPI-PLC (7,47-49) resulting in a sustained calcium influx necessary for completion of exocytosis (50,51). The sustained Ca^{2+} elevation appears to result from the release of Ca^{2+} from an IP_3-sensitive intracellular store (the acrosome). Emptying of stores would activate plasma membrane store-operated channels. The membrane store-operated channels that mediate the sustained phase of ZP3-evoked calcium entry could be one or more of the members of the transient receptor potential (TRP) cation channel family. The mechanisms that link PPI-PLC action to the opening of TRP channels have not yet been resolved but may involve, in addition to IP_3 emptying of stores and activation of store-operated (TRP) channels, a direct activation of TRP channels by DAG (42).

In any case, the mechanisms regulating early Ca^{2+} influx and sustained Ca^{2+} elevation in spermatozoa are still poorly defined. Clarification of these mechanisms would benefit from noting recent work arguing against the use of thapsigargin to deplete Ca^{2+} stores, as it also induces a cell stress response (41), and studies identifying the role of PKC-independent, DAG-mediated, regulation of transient receptor potential (TRP) channels (42).

Studies in recent years have helped to identify various types of PLCs present in spermatozoa, their regulation and their possible involvement in DAG generation during acrosomal exocytosis. Six classes of phosphoinositide-specific PLC isozymes have been identified, and each class includes more than one type of isozyme. They are: PLC-beta-1, -2, -3 and -4, PLC-gamma-1 and -2, PLC-delta-1, -3, and -4, PLC-epsilon, PLC-nu-1 and -2, and PLC-zeta (52-55). A PLC-delta-2 earlier found

in bovine tissues is now considered a homologue of mouse and human PLC-delta-4 (56). Of these isozymes, evidence for the following PLC isozymes exists for spermatozoa: PLC-beta-1 and -3, PLC-gamma-1 and -2, PLC-delta-4, and PLC-zeta (37,47-49,57-60). Of these isozymes, PLC-zeta is now recognized as the sperm factor responsible for triggering Ca^{2+} oscillations in the egg after fertilization (54,61) and it is not yet know whether it has any role in sperm function before fertilization.

Evidence for PIC-gamma activation, an isozyme modulated by tyrosine phosphorylation, has been presented for mouse spermatozoa (18,47). Immunostaining indicated that PLC-gamma-1 was restricted to the sperm head, with capacitation inducing translocation of the enzyme from the soluble to the particulate fraction (47). The enzyme's activity was increased in ZP-treated cells and this effect was prevented by tyrphostin a treatment that also suppressed ZP-induced acrosomal exocytosis (62).

There is no direct evidence of activation of PLC-beta (which in somatic cells seems to be activated by pertussis toxin-insensitive GTP-binding proteins G_q and G_{11}) upon stimulation of spermatozoa, but both PIC-beta-1 and -3 (37,48) and $G\text{-}_{alpha\text{-}q/11}$ have been identified by Western blotting and localized to the acrosomal region of mouse spermatozoa (37). The possible participation of PLC-beta in acrosomal exocytosis may been inferred from the observation that progesterone-stimulated DAG formation is not blocked by pertussin toxin (63). Studies on spermatozoa from mice in which the PLC-beta-1 gene has been knocked out appear to suggest that this isozyme participates in acrosomal exocytosis (64) but more studies are needed to further characterize the role of the beta isozymes.

Spermatozoa may have a PPI-specific PLC activated by a pertussis toxin-sensitive GTP-binding protein (G_o or G_i type) since DAG formation stimulated by ZP was blocked by pertussis toxin (63) and, furthermore, activation of G_i proteins has been demonstrated after ZP stimulation of mouse spermatozoa (65). No clear information exists as to the identity of the PLC regulated by GTP-binding protein.

It has been predicted that spermatozoa may contain a PPI-specific PLC that is activated by an elevation of intracellular Ca^{2+} (66). It is now known that PLC-delta-4 is the most Ca^{2+} sensitive of the PLCs and its presence in spermatozoa has been reported (48,49). Its activation during ZP-induced acrosomal exocytosis seems essential because PLC-delta-4 null mutant male mice are sterile or have severe infertility. Acrosomal exocytosis does not take place after treatment of mutant spermatozoa with ZP (48) or with progesterone (49). Detailed studies on temporal and spatial distribution of intracellular Ca^{2+} rises in spermatozoa revealed that PLC-delta-4 null mice showed no or very little elevations of intracellular Ca^{2+} after ZP or progesterone (incidentally, the progesterone concentration employed in this work, 100 µM, was very high and the lack of total inhibition after progesterone treatments in null mice could be due to this fact). After thapsigargin or A23187 treatments, there was also a reduction in intracellular Ca^{2+} in null mice and it has been concluded that PLC-delta-4 function may be related to mobilization of Ca^{2+} from stores before Ca^{2+} entry (49). This is not in agreement with the view that PLC would be activated by the initial Ca^{2+} entry occurring via activation of T-type, low-voltage activated channels (43,44) and further studies on PLC-delta-4 null mice should address these discrepancies.

A PLC from human spermatozoa specific for the PPIs has been partially purified and characterized (67). It was found to hydrolyze phosphatidylinositol, PIP and PIP_2 and to be maximally activated in the presence of 5 mM Ca^{2+}. However, one half of maximal activity was still observed without any Ca^{2+} addition indicating that endogenous Ca^{2+} 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^{2+} requirements in live spermatozoa stimulated with A23187 in the presence of different concentrations of Ca^{2+} or EGTA (32). 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 (67). A PPI-specific PLC was also purified from bull sperm (68) 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 (32). 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 (26). 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 (25). In parallel experiments, exocytosis triggered by progesterone was blocked by neomycin or U73122, again with U73343 having no effect.

3.2. Phosphatidylcholine-specific PLC

In somatic cells the main route for DAG formation from phospholipids other than the phosphoinositides appears to involve the concerted action of PLD and PPH (69,70), and the same has been assumed for mammalian spermatozoa (71). However, the possible role of a PC-specific PLC (PC-PLC) in the generation of sustained levels of DAG has also been recognized in somatic cells (72,73) and its role in signal transduction has been described, for example, in response to tumour necrosis factor (74-76) or other stimuli (77). 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 alkyl-acyl-PC, with a concomitant rise in DAG or alkyl-

acyl-glycerol and no or very little subsequent changes in PA or alkyl-PA. These responses are indicative of PC-PLC activity (7,25,63). This agrees well with an earlier study showing that mammalian spermatozoa have a PC-specific PLC localized to the acrosomal region (78). This PC-PLC has been purified from seminal plasma and shown to generate DAG from [^{14}C]dioleoyl-PC, but not from similarly labelled phosphatidylinositol or phosphatidylethanolamine (78); this is coincident with the source of DAG identified in a study in which sperm phospholipids were labelled with radioactive precursors (29). Thus, in mammalian spermatozoa, DAGs are being generated directly by PLC-mediated hydrolysis of PC.

Other studies have also detected PLC activity, not related to hydrolysis of phosphoinositides, in rabbit, boar, bull, and mouse spermatozoa (79-82). However, some of these results should be evaluated critically because conditions used for assays not always allowed discrimination of PC-specific acitivity, 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) (82).

Very little PA seems to be formed from the DAG that is generated by PC-PLC (7,25,63). 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.

In comparison to what is known about PPI-specific PLC, very little is known about PC-specific PLC in somatic or germ cells (although a great deal more is known about bacterial PC-PLCs) and further studies are needed to advance this area.

4. PHOSPHOLIPASE D

There is clear evidence that PLD plays an important role in signalling during the acrosome reaction of sea urchin spermatozoa (83). 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 very little generation of PA upon stimulation and in no case was PA generated before DAG (7,25,27,84). PLD activity was also examined using the ability of this enzyme to catalize a transphosphatidylation reaction in the presence of ethanol (a reaction that unambiguously identifies PLD activity; 70) and which results in the formation of phosphatidylethanol at the expense of PA. Very little formation of phosphatidylethanol was observed in spermatozoa from ram, mouse or man upon stimulation (7,25,84). Changes in 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 (84). 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 (84). 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 the occurence of acrosome reactions (84).

Immunodetection studies (Western blots and immunocytochemistry) have revealed the presence of a 115-kDa PLD in the acrosomal region of bovine spermatozoa (85). Furthermore, it seems that PLD is translocated from the cytosolic to the particulate fraction when cells are stimulated with agonists that activate PKC (85). Based on these results, it has been speculated that PLD could be translocated to the outer acrosomal membrane (a component of the particulate fraction), where the enzyme would release the fusigenic PA that may help promote membrane fusion (85). In any case, no additional evidence has been presented so far demonstrating that PLD is actually activated or leads to the release of PA or other metabolites and, therefore, has a role during acrosomal exocytosis in mammalian sperm. Future studies should be carried out to sustain this hypothesis.

On the other hand, evidence suggesting a possible role of PLD during capacitation has been presented (86). This evidence is based on the finding that butan-1-ol (but not butan-2-ol), or a ceramide, block responses related to the acquisition of a capacitated state such as F-actin formation or induction of acrosome reactions by the ionophore A23187 (markers of capacitation in the experimental system used). The latter is difficult to reconcile with the fact that spermatozoa can undergo acrosome reaction in response to A23187 even if they are not capacitated (32). In addition, a role for PLD during capacitation is also based on the slight increase in PLD activity (measured using a transphosphatidylation assay with butan-1-ol) during incubation under capacitating conditions, although additional evidence (e.g. effects of exogenous phosphatidic acid, or PLD) could have other explanations and have not been ruled out.

5. PHOSPHOLIPASE A_2

Phospholipase A_2 (PLA_2) acts on membrane phospholipids releasing the fatty acid in position 2 of the glycerol backbone thus generating a lysophospholipid (Figure 1). Both metabolites have important roles in sperm

cell function either as second messengers or as metabolites used for the generation of further messenger molecules (87). The action of PLA_2 on PC generates lysoPC and fatty acids such as arachidonic acid, whereas PLA_2 hydrolysis of other phospholipids, such as phosphatidylethanolamine (PE) or phosphatidylinositol (PI), would yield lysoPE or lysoPI, respectively. These other lysophospholipids may also have important roles as messengers or metabolites (87). Furthermore, arachidonic acid is only one of the fatty acids present in position 2 of PC, PE or PI. Other unsaturated fatty acids (such as oleic, linoleic or linolenic acid, as well as docosahexaenoic acid) are also present in position 2 of these phospholipids (88). Upon release by the action of PLA_2, they too could have a biological function. In addition, phospholipids in sperm membranes are not only diacyl lipids; they could also be alkyl-acyl-phospholipids (88) and in this case the action of PLA_2 would generate an alkyl-lysophospholipid.

5.1. Types of PLA_2 in spermatozoa

PLA_2 constitutes a large superfamily of enzymes (89-91,92a,93). Several mammalian PLA_2s have been recognized and they have been grouped in four main categories based on their cellular localization, size, Ca^{2+} requirement and/or substrate preference. A classification system based on structure similarities has also been proposed (89-91). The four main PLA_2 types are: (a) secreted, low molecular weight enzymes (14-19 kDa or 55 kDa) which, in general terms, require millimolar levels of Ca^{2+}. They are generically called secreted PLA_2 ($sPLA_2$) and various groups are recognized in mammals, namely IB, IIA, IIC, IID, IIE, IIF, III, V, X XII; (b) the cytosolic PLA_2s ($cPLA_2$) consists of three enzymes ($cPLA_2$-alpha, -beta, and -gamma, or group IVA, IVB and IVC, respectively); they haver larger sizes (85, 110 and 60 kDa, respectively) and require micromolar Ca^{2+} (IVA, IVB), or no Ca^{2+} (IVC); (c) the Ca^{2+}-independent PLA_2s ($iPLA_2$s) with two enzymes in mammals ($iPLA_2$-beta and -gamma, or group VIA and VIB, respectively). They have high molecular weights (85-88kDa and 90 kDa respectively) and they require no Ca^{2+}; and (d) a group of enzymes that is distinguished by their ability to hydrolyze, and thus inactivate, platelet-activating factor (PAF); these enzymes are collectively called PAF-acetylhydrolases (groups VIIA, VIIB, VIIIA, VIIIB). They have intermediate sizes (30-45 kDa) and no requirement for Ca^{2+}.

There is, unfortunately, little information on which are the PLA_2 isozymes present in mammalian spermatozoa although some recent studies have started to shed light on this question. Efforts have been mainly directed towards a biochemical characterization of PLA_2 in spermatozoa from various species and information gathered so far agrees on the fact that the predominant sperm PLA_2 is maximally stimulated in the presence of millimolar levels of Ca^{2+}, although PLA_2 activity can also be detected in the absence of Ca^{2+} in *in vitro* assays (92). It should nevertheless be noted that most studies carried out on mammalian spermatozoa have extracted PLA_2 using acid extraction and, thus, that any possibility of detecting high molecular weight cytosolic isoenzymes has been lost with this procedure (93b). *In vivo*, maximal activity after cell stimulation is again seen in the presence of millimolar Ca^{2+} with no activity if extracellular Ca^{2+} is chelated (87). Nevertheless some PLA_2 activity may still exist in low or no Ca^{2+} conditions because sperm phospholipids can be labelled with radioactive arachidonic acid in medium containing EDTA (94), suggesting that there is an active deacylation by PLA_2 and subsequent reacylation.

Partial purification and characterization of a human sperm PLA_2 has revealed the existence of an enzyme of about 14-16 kDa. Unfortunately only limited sequence data has been presented and it is thus difficult to asign this sperm PLA_2 isozyme to any of the recognized groups (90). In any case, partial sequencing of the N-terminal region of this PLA_2 has shown some similarities with secreted PLA_2s of groups I and II (from snake venom, and porcine/human pancreas), although this human sperm PLA_2 appears to represent a novel sequence (95). A study using antibodies raised against cobra (*Naja naja*) venom (presumably group IA PLA_2) identified a 16 kDa protein in SDS-extracts from bull spermatozoa (96). Similarly, antibodies against porcine pancreas PLA_2 (group IB PLA_2) recognized a protein in hamster and human sperm (97,98). These antibodies have been used in immunolocalization work; PLA_2 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_2 are capable of inhibiting acrosomal exocytosis in hamster spermatozoa (99).

The expression of a group IIC PLA_2 has been identified in various spermatogenic cells in the mouse; this PLA_2 is expressed only in testis. Expression of the *Pla2g2c* gene has been detected in mouse pachytene spermatocytes, secondary spermatocytes and round spermatids (100). It is therefore possible that this PLA_2 is present in mature mouse spermatozoa but differences may exist between species because, in humans, *PLA2G2C* appears to be a nonfunctional pseudogene (101). Recent studies have considerably advanced the identification of low molecular weight secreted PLA_2s isozymes present in spermatozoa (102). The presence of group IIC PLA_2 in mouse spermatozoa has been confirmed, and PLA_2s of groups IID, IIE, IIF, V, and X have been identified in mature sperm cells. PLA_2-IIA was not identified because the C57BL/6 mice used for this study are a strain with a natural disruption of this gene (103). More detailed analyses revealed that PLAs IIE, V and X are located in the acrosome of mouse spermatozoa. Interestingly the expression of these isozymes vary, with some (e.g. PLA_2-IIC and -X) being expressed from the early stages of spermatogenesis whereas others (PLA_2-IIE and -V) are seen in spermatocytes and later stages and yet other isozymes (e.g. PLA_2-IID and -IIF) are only detected in spermatids (102). Similar results were obtained in human spermatozoa when the profiles of PLA_2s isozymes were examined in the testis and epdidiymis, with the exception of PLA_2-IIC, which is not expressed in humans.

In somatic cells, a cytosolic PLA_2 ($cPLA_2$-alpha, group IVA PLA_2) is activated during exocytosis. There is

no indication as to whether this isozyme is present in mammalian sperm. Interestingly, experiments in which the gene coding for cPLA$_2$-alpha has been mutated showed that homozygous male mice with the mutations had no impairement in their fertility (104,105), suggesting that this cPLA$_2$ may not be essential in events underlying sperm function, including exocytosis. Furthermore, these mutated mice were generated using the C57BL/6 strain which naturally lacks a secreted PLA$_2$ (PLA$_2$-IIA) which would suggest that this other enzyme is also not required for acrosomal exocytosis (106).

Recent work has identified a mouse cPLA$_2$-gamma (group IVC PLA$_2$) that localizes solely to the ovary (107), contrasting with human cPLA$_2$-gamma that is present only in the heart and skeletal muscle. This suggests that this enzyme does not appear to be present in testis and, hence, in spermatozoa.

Studies on iPLA$_2$-beta (group VIA) null mice have revealed that this enzyme may have important roles in spermatozoa (108). This enzyme shows high expression levels in testes from wild-type mice, but testes from null mice are not defficient in arachidonic acid-contaning glycerophosphocholine lipids suggesting that this enzyme does not have an essential role in formation of these lipids in testis. However, spermatozoa from null mice have reduced motility and impaired ability to fertilize oocytes both *in vitro* and *in vivo*. In agreement with this, bromoenol lactone (an iPLA$_2$-beta inhibitor) reduced motility of wild-type mouse spermatozoa. It remains to be established whether this enzyme has any role in acrosomal exocytosis.

Studies on PLA$_2$ in reproductive tissues have identified and characterized PLA$_2$ activities in bull prostate, seminal vesicle, Cowper's gland and in seminal plasma (109,110) and in human seminal plasma (111). Antibodies generated against PLA$_2$ purified from bovine seminal vesicle fluid blocked PLA$_2$ activity in bovine seminal plasma, seminal vesicles and its fluid, and gave single precipitation lines with the same samples, but no crossreactivity was detected with other reproductive tissues or human seminal plasma (112). Using this antibody, PLA$_2$ 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 postacrosomal region, and the midpiece) but it was not present on the surface of epididymal spermatozoa (112). These results suggest that different PLA$_2$s may be present in seminal plasma, and that PLA$_2$s may become bound to the surface of spermatozoa upon ejaculation. This may not be true for other species such as the mouse, where surface-active PLA$_2$ was found in epididymal spermatozoa (113) or it could suggest that surface PLA$_2$ would be contributed by the epididymis.

The PLA$_2$ identified in bull reproductive tissues has been purified and characterized (109). The PLA$_2$ activity detected in human seminal plasma, which reacted with monoclonal antibodies raised against human synovial fluid PLA$_2$ (111), 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$_2$. A novel PLA$_2$ from bull seminal plasma has also been purified (114) and found to be a 60 kDa enzyme. It has been later shown to be a platelet-activating-factor acetylhydrolase, although it is capable of hydrolysing long-chained phosphatidylcholine (115). Since these enzymes can become bound to the surface of spermatozoa it would be interesting to test how much of the PLA$_2$ activity detected in spermatozoa relates to PLA$_2$s of seminal plasma that are bound to the sperm surface.

5.2. Sperm PLA$_2$ - Biochemical data

Probably the majority of the *in vitro* studies carried out so far have analyzed (although not deliberately) the activity of the low molecular weight PLA$_2$, since methods used for exactraction of enzyme activity involved acid treatment and this leads to inactivation of the high molecular weight cytosolic isoform (93b). Studies on sperm PLA$_2$ have shown that in all mammalian species examined so far the activity of this enzyme is Ca^{2+}-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 (92,113,116).

PLA$_2$ from ejaculated bull spermatozoa assayed *in vitro* can hydrolyse linolelic- and arachidonic-PE, as well as linolenic- and arachidonic-PC (and also a fluorescent derivative of PC) (109). Ram sperm PLA$_2$ assayed *in vitro* hydrolyses both PE and PC but it appears to prefer PE as substrate over PC (117). 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 (88).

It has been found that sperm PLA$_2$ activity can be inhibited by compounds known to affect PLA$_2$ from other cellular systems. However, PLA$_2$ 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$_2$ activity (113,116,118,119) but does not affect ram (92) or human sperm PLA$_2$ (116; but see 120). On the other hand, p-bromophenacyl bromide (pBPB) inhibits the activity of PLA$_2$ from all the species examined so far: golden hamster, guinea pig, mouse and man (113,116,118-120). Finally, dexamethasone, although capable of inhibiting PLA$_2$ from various cells (121,122) including ram spermatozoa (92), does not inhibit mouse sperm PLA$_2$ (113). The reasons why sperm PLA$_2$ differ in sensitivity to classic, though probably less specific inhibitors remain obscure. We have examined the sensitivity of ram sperm PLA$_2$ to more specific compounds such as chloracysine and the two alkylamines Ro-4493 and Ro-4639 (92) both in *in vitro* assays and in labelled, stimulated cells. Enzyme activity in sperm sonicates was completely abolished by these reagents in a fashion similar to their effects on PLA$_2$ from porcine pancreas or human neutrophil cytoplasts (123,124). *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 (87).

5.3. Activation and role of PLA_2 during acrosomal exocytosis

Various early studies have gathered evidence suggesting that PLA_2 may be involved in sperm acrosomal exocytosis. However, most studies have only contributed indirect evidence: (a) spermatozoa from several species (hamster, guinea pig, man) treated with putative PLA_2 inhibitors (mepacrine or pBPB) failed to undergo an acrosome reaction either spontaneously or in response to ionophore treatment (119,125-127); and (b) spermatozoa treated with exogenous metabolites generated by PLA_2 (i.e. lysophospholipids or fatty acids) experienced a stimulation/acceleration of acrosomal exocytosis (128-130). None of these studies have actually quantified activity of PLA_2 (e.g. by labelling lipid pools in cells and measuring fatty acid release) and therefore do not provide direct evidence for PLA_2 participation in exocytosis. There has been a preliminary study labelling human sperm with radioactive arachidonic acid, and then follow changes upon treatment with A23187 (131). In this study, 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.

A series of subsequent studies have supplied thorough evidence demonstrating that PLA_2 plays an essential role in the release of fatty acids and lysophospholipids involved in sperm membrane fusion during acrosomal exocytosis (87). Using ram spermatozoa as model species, it was possible to label sperm phospholipids using radioactive [^{14}C]arachidonic acid or [^{32}P]P_i as precursors. Stimulation of Ca^{2+} entry with the ionophores A23187 or ionomycin revealed a time- and concentration-dependent increase in free fatty acids. In cells labelled with [^{32}P]P_i, a parallel rise in lysoPC was detected. This, together with the fact that no DAG-derived arachidonic acid release was observed, clearly indicates PLA_2 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_2 in exocytosis derives from the finding that the PLA_2 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 (87). Similarly, the PLA_2 inhibitor aristolochic acid blocked A23187-induced acrosomal exocytosis which was restored by the inclusion of arachidonic acid or lysoPC (132,133). These results provided very strong evidence to suggest that PLA_2 may have an essential role in the acrosome reaction.

Additional, more recent studies have concentrated on the activation and regulation of PLA_2 in response to the natural agonists progesterone and ZP (8,134), following up from some early work on progesterone stimulation of human (135) and boar spermatozoa (136). In guinea pig spermatozoa preincubated with [^{14}C]arachidonic acid or [^{14}C]choline chloride to label phospholipid pools, stimulation with progesterone or ZP led to release of arachidonic acid or lyso PC and a parallel decrease in PC (8,134). Similar results were observed when guinea pig spermatozoa were stimulated with GABA (137). These lipid changes, which are indicative of PLA_2 activity, did not occur if spermatozoa were pre-exposed to the PLA_2 inhibitor aristolochic acid (8,134,137). Exposure of spermatozoa to submaximal concentrations of both progesterone and ZP resulted in a synergistic increase of arachidonic acid and lysoPC releases, and exocytosis (8), suggesting that, under natural conditions, both agonists interact to bring about PLA_2 activation during acrosomal exocytosis.

5.4. Regulation of sperm PLA_2

Extensive work has been done on the characterization of mechanisms regulating PLA_2 activity in many cells and it has been found that these mechanisms differ between cell systems. Evidence has been gathered in favour of several mechanisms of PLA_2 regulation: (a) G-protein mediated PLA_2 activation, (b) activation of the enzyme through a rise in intracellular Ca^{2+}, (c) activation via phosphorylation by mitogen-activated protein (MAP) kinase, and (d) modulation of PLA_2 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_2, in some cells DAG appears to stimulate PLA_2 directly without the participation of the PKC pathway (138-141). It has been argued that since some PKCs and PLA_2s contain similar regulatory sequences (142), DAG may bind directly to PLA_2 and activate it, in a way similar to its mode of action on PKC (143). In spermatozoa little is still known about the mechanisms that regulate the activation of PLA_2, and the regulation of the different PLA_2 isoforms.

One possible mechanism regulating PLA_2 in spermatozoa is that involving G proteins (144). It is not clear whether G proteins are directly related to PLA_2 activation or, more likely, whether G proteins activate a signalling pathway, such as Ca^{2+} influx, or PIC mediated DAG generation, that eventually results in PLA_2 activation.

Another possible pathway modulating PLA_2 is the DAG-PKC-MAP kinase cascade (145). The direct activation of PLA_2 by PKC has been postulated (71) but, so far, there is no evidence for this interaction *in vivo* (146). On the other hand, MAP kinase can also be phosphorylated by the cAMP-PKA pathway (147) and this could be one additional mechanism modulating PLA_2 in spermatozoa (148).

Evidence for PKC-mediated phosphorylation of a variety of substrates has now been presented for human spermatozoa stimulated with progesterone (29,149). In addition, components of the MAP kinase pathway, such as Ras and ERK 1 and 2 (150-153), have been identified in

spermatozoa. MAP kinase activation in reponse to A23187 or progesterone has also been demonstrated in human spermatozoa (151,154). However, there is contradictory evidence in relation to the regulatory role of MAP kinase during acrosomal exocytosis in spermatozoa (154) with some studies failing to detect evidence (151,154) and others supporting it (152,155,156).

We have recently carried out several studies to understand mechanisms regulating PLA_2 activation during acrosomal exocytosis (8,137). Progesterone-induced PLA_2 activation appears to be mediated by a $GABA_A$-like receptor because bicuculline (a $GABA_A$ receptor antagonist) blocked arachidonic acid release and exocytosis. In agreement with this, GABA mimicked progesterone actions. On the other hand, ZP-induced activation of PLA_2 seemed to be transduced via G_i proteins because pertussis toxin blocked arachidonic acid release and acrosomal exocytosis (8).

Agonist-stimulated PLA_2 activation may be regulated by DAG. This was tested using a DAG kinase inhibitor, which is known to stimulate accumulation of DAG when spermatozoa are treated with A23187 (26) or progesterone (25). Inclusion of the DAG kinase inhibitor when ram spermatozoa were treated with A23187 (30), or when guinea pig spermatozoa were stimulated with ZP (134) led to an enhancement of both PLA_2 activity and exocytosis. Further evidence for DAG regulation of PLA_2 was gathered in experiments in which permeable DAGs were added to intact labelled cells. Under these conditions, PLA_2 activity and exocytosis were enhanced in relation to sperm cells not exposed to exogenous DAGs (30).

Sperm PLA_2 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_2 in an *in vitro* enzyme assay (92). In addition, alkyl-acyl-gycerols can also enhance the activity of the enzyme *in vitro* (157), suggesting that a variety of PC-derived diglycerides could modulate PLA_2. The stimulatory effect of exogenous diglycerides was evident when either 1,2- or 1,3-DAGs were included, and similar stimulatory effects were also seen with alkyl-acyl-glycerol (30). This indicates that the action of the diglycerides, at least in part, is not mediated by PKC, since neither 1,3-DAG nor alkyl-acyl-glycerol are capable of activating the kinase.

DAG action may nevertheless be mediated to a great extent by PKC because release of arachidonic acid and exocytosis stimulated by progesterone or ZP were blocked by the PKC inhibitors staurosporine or chelerythrine chloride (8). PLA_2 could also be regulated by the cAMP-PKA pathway since inclusion of the PKA inhibitor 14–22 amide or H-89 led to a reduction in arachidonic acid release and exocytosis after progesterone or ZP (8). Finally, it is also possible that a MAP kinase pathway is involved in PLA_2 activation and acrosomal exocytosis. GABA-, progesterone- and ZP-induced release of arachidonic acid and exocytosis were prevented by the MAP kinase kinase (MEK) inhibitors U0126 and PD98059 but not by the inactive analogue U0124 (137). Taken together, our results suggest that PLA_2 plays a fundamental role in agonist-stimulated exocytosis and that various intracellular signalling mechanisms have important roles in its regulation.

5.5. Roles of lysophospholipids and fatty acids

The metabolites generated by PLA_2 seem to serve as co-activators of some types of PKC (158,159), but their major role could relate to perturbation of cell membranes during fusion and this has been extensively investigated in mammalian spermatozoa.

Lysophospholipids such as lysoPC may play a role in acrosomal exocytosis and this has been suggested by some early studies in which pretreatment of guinea pig spermatozoa with lysoPC shortened capacitation and resulted in a rapid induction of acrosomal exocytosis after addition of millimolar Ca^{2+} (128). Additional studies have confirmed that lysoPC triggers the reaction in spermatozoa (hamster: 160,161; bull: 162). In spermatozoa in which exocytosis is induced by the ionophore A23187, lysoPC exerts a concentration-dependent enhancement of exocytosis (87). Further evidence for its role in exocytosis comes from experiments in which PLA_2 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: 87,132,133; hamster: 161). It is interesting that not all lysophospholipids have a stimulatory role on exocytosis. Thus, lyso PC and lysoPI were capable of enhancing acrosomal exocytosis, whereas lysophosphatidylserine (lysoPS) was not (87,161). In fact, lysoPS appears to have an inhibitory role under certain conditions (161).

Fatty acids may be involved in membrane perturbation. Early work has indicated that certain fatty acids stimulated acrosome reactions in precapacitated golden hamster or guinea pig spermatozoa (130,163), whereas more recent studies have also shown that arachidonic acid is able to enhance the response to A23187 in ram speramatozoa (87). Although various unsaturated fatty acids may have a fusigenic role (164), it is now evident that some fatty acids (such as arachidonic acid) may be further metabolized and could act through derived metabolites. Support for this idea comes from (a) experiments where spontaneous acrosome reactions were partially or totally blocked by the addition of inhibitors of cyclo- or lipoxygenase pathways, and (b) the ability of arachidonic acid-derived metabolites to stimulate the acrosome reaction (165-167). This evidence is, in general terms, circumstantial since levels of arachidonic acid or metabolites generated by either pathway have not been measured. One study (166) has presented some evidence of production of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), the product of 15- lipoxygenase activity, after stimulation of bull spermatozoa with A23187 and Ca^{2+}. However, these results will have to be reevaluated since it has been found that prostasomes have 15-lipoxygenase activity that could be responsible for generation of 15-HETE (168). Another study has provided evidence for an additional role of arachidonic acid derived metabolites,

since prostaglandin E has been found to act on a novel human sperm receptor coupled to pertussis toxin-insensitive G-alpha-q/11 which is involved in Ca^{2+} influx (169). The biological significance of this mechanism remains to be established.

6. PERSPECTIVE

Studies carried out in the past have provided information on the possible involvement of phospholipases in signalling mechanisms underlying acrosomal exocytosis in mammalian spermatozoa. Nevertheless, much work is still needed in order to characterize which PLC and PLA_2 isozymes are actually present in spermatozoa and which mechanisms are responsible for regulating their activities. In addition, future work should try to characterize the potential targets for the lipid messengers generated by these phospholipases and the possible interactions with other intracellular signalling pathways, particularly those involving phosphorylation cascades.

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Abbreviations: DAG: 1,2-diacylglycerol, ERK: extracellular signal-regulated kinase, GABA: Gamma-aminobutyric acid, IP_3: 1,4,5-trisphosphate, MAG: monoacylglycerol, MAP kinase: mitogen-activated protein kinase, MEK: MAP kinase (ERK) kinase, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanol, PI: phosphatidylinositol, phosphoinositide, PIC: phosphoinositidase C, PIP: phosphatidylinositol 4-phosphate, PIP_2: phosphatidylinositol 4,5- bisphosphate, PKA: protein kinase A, PKC: protein kinase C, PLA_2, phospholipase A_2, PLC: phospholipase C, PLD: phospholipase D, PPI: polyphosphoinositide, ZP: zona pellucida

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