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INTRACELLULAR EVENTS AND SIGNALING PATHWAYS INVOLVED IN SPERM ACQUISITION OF FERTILIZING CAPACITY AND ACROSOME REACTION

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1. ABSTRACT

Two processes, namely capacitation and acrosome reaction, are of fundamental importance in the fertilization of oocyte by spermatozoon. Physiologically occurring in the female genital tract, capacitation is a complex process, which renders the sperm cell capable for specific interaction with the oocyte. During capacitation, modification of membrane characteristics, enzyme activity and motility properties of spermatozoa render these cells able to penetrate oocyte investments and responsive to stimuli that induce acrosome reaction prior to fertilization. Physiological acrosome reaction occurs upon interaction of the spermatozoon with the zona pellucida protein ZP3. This is followed by liberation of several acrosomal enzymes and other constituents that facilitate penetration of the zona and expose molecules on the sperm equatorial segment that allows fusion of sperm membrane with the oolemma. The molecular mechanisms and the signal transduction pathways mediating the processes of capacitation and acrosome reaction have been partially defined, and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodeling in sperm plasma membrane as well as changes in protein phosphorylation. Some of the kinases and phosphorylated proteins that are involved in the processes of capacitation and acrosome reaction have been now characterized. Characterization of sperm receptors to physiological inducers of acrosome reaction is in progress. This review summarizes the main signal transduction pathways involved in capacitation and acrosome reaction. Furthermore, the mechanisms underlying sperm DNA fragmentation are also briefly reviewed.

2. SPERM CAPACITATION: BACKGROUND

The process of capacitation consists of a series of functional biochemical and biophysical modifications that render the ejaculated spermatozoa competent for fertilization of the oocyte. This fundamental process normally takes place in the female genital tract during the migration of spermatozoa to the site of fertilization as a consequence of specific interactions between sperm and epithelial tubal cells (1). However, under appropriate conditions, capacitation can be also induced in vitro (1). Most of our knowledge regarding this process has been derived from in vitro studies. Following ejaculation, sperm surface is surrounded by molecules, known as decapacitation factors (DF), that, until released, keep the sperm in a non-capacitated state. These factors associate with spermatozoa following contact with seminal fluids and are progressively released from sperm surface during capacitation. DF thus modulate capacitation leading spermatozoa to the maximal fertilizing ability at the site of fertilization (2). It has been supposed that DF, once attached to the sperm surface, activate an intracellular Ca^{2+}-ATPase maintaining low intracellular calcium concentration ($[Ca^{2+}]i$) (3) and when they are released from the surface $[Ca^{2+}]i$ increases. Several molecules have been indicated as possible DF (1).

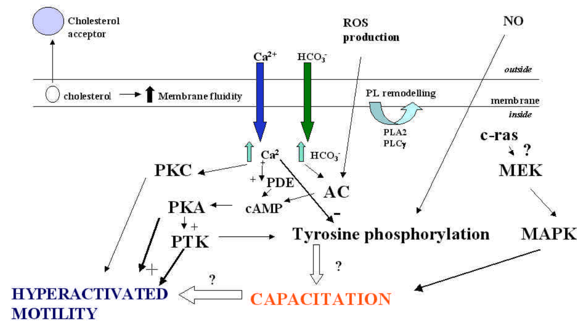

Figure 1. Schematic representation of the main events occurring under conditions leading to capacitation and development of hyperactivated motility of human spermatozoa in vitro. Changes in membrane permeability to several ions have been described, among these Ca^{2+} and bicarbonate (HCO3-), whose influx increase during capacitation, have been reported to exert a primary role in the process. Membrane fluidity increases due to the loss of cholesterol from the membrane which may be accelerated by the presence of a cholesterol acceptor in the external medium. Remodeling of sperm membrane phospholipids (PL) and activation of phospholipases (PLA2 and PLCγ1) have also been shown: in particular, increased synthesis of phosphatidylcholine from phosphatidyl-ethanolamine, phosphatidylinositol and lyso-phosphatidylcholine have been documented. A time-dependent increase of tyrosine phosphorylation of proteins is associated with development of capacitation. The increase of tyrosine phosphorylation is primarily dependent on the increase in bicarbonate, which, in turn, activates adenyl cyclase (AC) with increased generation of cAMP and subsequent activation of protein kinase A (PKA). PKA activation leads to the activation of sperm tyrosine kinase(s) (TK). On the contrary, Ca^{2+} inhibits tyrosine phosphorylation during capacitation. Other possible physiological modulators of tyrosine phosphorylation during capacitation are reactive oxygen species (ROS) that may be generated from spermatozoa or leucocytes present in the ejaculate and nitric oxide (NO). An involvement of protein kinase C (PKC) and ras-MEK-MAPK (mitogen-activated protein kinase) pathways has also been reported.

We have recently demonstrated that two proteins present in seminal plasma, uteroglobin and transglutaminase, inhibit sperm capacitation and motility, thus representing two possible DF candidates (4). Another sperm inhibitory factor present in seminal fluid is cholesterol, which is known to inhibit several sperm functions, including capacitation (5,6). In seminal plasma there are also molecules that can stimulate fertilizing ability of spermatozoa such as fertilization-promoting peptide (FPP). FPP is a small peptide that promotes capacitation (7) and inhibits spontaneous acrosome loss retaining fertilization potential of sperm until the site of fertilization is reached. The role of FPP in sperm physiology has been subject of a recent review (8).

Capacitation is associated with development of a distinct motility pattern called hyperactivation (1), which is characterized by pronounced flagellar movements, marked lateral excursion of the sperm head and a non linear trajectory. Whether the development of hyperactivated motility is related to the biochemical and biophysical changes occurring during the process of capacitation is still matter of debate. It is worth noting that signal transduction mechanisms involved in the development of this special sperm motility pattern are similar to those described to occur during capacitation (see later). It has been recently shown that, during in vitro capacitation, modifications of mitochondrial morphology, which may be relevant for the development of the hyperactivated motility pattern, also occur (9). An additional manifestation of sperm capacitation is the acquisition of the ability to undergo acrosome reaction in response to physiological stimuli such as the zona pellucida protein 3 (ZP3) and progesterone (P). The responsiveness of spermatozoa to ZP3 (10), P (11) and other stimuli of acrosome reaction increases during capacitation, assuring maximal responsiveness at the site of fertilization. Capacitation involves modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activities and modulation of expression of intracellular constituents (1). These events are made possible by activation of a cascade of signaling pathways (schematized in Figure 1) effected by unknown mediators during transit of sperm in the female reproductive tract or during incubation in vitro in defined media. All these pathways may then cross-talk among each other determining the development of capacitation. However the exact relationship among these modifications is not yet completely understood. In addition, there is as yet no well defined method that allows distinction of capacitated from noncapacitated spermatozoa. Capacitation does not occur synchronously in spermatozoa (12). In addition, capacitation is transient and already capacitated spermatozoa cannot be capacitated again (12). These complexities in in vitro capacitation makes it difficult to appropriately interpret in vitro studies. This review mainly focuses on three aspects of molecular changes that occur during capacitation: concentration of intracellular calcium and other ions, changes in lipid distribution and composition and changes in protein phosphorylation and kinases activities.

2.1. Modification in concentration of intracellular calcium and other ions during capacitation

Modification of intracellular concentration of calcium ions ($[Ca^{2+}]i$) is the most fully characterized biochemical event during capacitation. An increase in the concentration of Ca^{2+} during capacitation has been demonstrated in several mammalian species (1) including human (13,14). Extracellular Ca^{2+} is indeed one of the necessary constituents for the completion of capacitation of spermatozoa in vitro (1). In spermatozoa $[Ca^{2+}]i$ is regulated by a Ca^{2+}-ATPase (acting as a Ca^{2+} extrusion pump) (15), Ca^{2+}/H^{+} exchanger system and Na^{+}/Ca^{2+} antiporter (acting as Ca^{2+} entrance systems) in the plasma membrane (16), and by putative intracellular Ca^{2+} stores, whose presence in human sperm has been suggested by several evidences (17-20). However, the role of intracellular calcium stores in the physiology of spermatozoa is still questioned (20-22). Recent data (19)

indicate that cytosolic Ca^{2+} is actively transported into the acrosome by an ATP-dependent, thapsigargin-sensitive pump and that may be released from the acrosome through an IP3-gated calcium channel. More recently, the existence of store-operated calcium channels that mediate sustained calcium increase in response to ZP3 in mouse sperm has been demonstrated (20). The location of such stores, however, remains to be demonstrated since endoplasmic reticulum is not present in mature spermatozoa and the acrosome does not appear, according to recent studies performed in individual spermatozoa, to retain significant amount of calcium (21,22). It has been hypothesized that modulation of the activity of the Ca^{2+}-extrusion system, in particular Ca^{2+}-ATPase, occurs during capacitation leading to an increase in intracellular Ca^{2+} (15,16). Drugs such as quercetin, that inhibit Ca^{2+}-ATPase, accelerate capacitation (15,16). A $Na+/Ca^{2+}$ exchanger is present in mammalian sperm, however, its role in controlling intracellular Ca^{2+} during capacitation is not clear. Similarly, although voltage-operated calcium channels (VOCC) have been demonstrated in mammalian spermatozoa (23), their role in regulating intracellular calcium concentration during capacitation is not clear. Moreover, although the presence of T-type VOCCT in germ cells has been unequivocally demonstrated (24,25) and their involvement in the effect of ZP3 on mature sperm indicated (24,20), the role of these channels in the process of capacitation is far from being defined. However, it seems quite clear that sperm VOCCT may be modulated during capacitation. Indeed, during this process sperm membrane becomes hyperpolarized due to enhanced K+ permeability (26). This hyperpolarization may act to prime VOCCT from an inactivated state to a closed one, which can be activated by an agonist inducing depolarization such as ZP3 (27). Downstream targets of calcium include the calcium-binding protein calmodulin, whose involvement in sperm capacitation has been recently demonstrated (28).

Besides Ca^{2+}, intracellular K^+ (29), Na^+ (30) and Cl- (16) concentrations have been shown to be modulated during capacitation. The increase of intracellular Na^+ appears to be important for capacitation, since the Na^+ ionophore, monesin, promotes this process in mouse sperm (16). The intracellular concentration in zinc ion decreases in the acrosome of hamster spermatozoa during capacitation (31). In addition, incubation of spermatozoa in a zinc-containing medium inhibits the process (31). These findings suggest that zinc may play a role in destabilization of plasma membrane during capacitation (31).

A rise in intracellular pH has been reported during capacitation of bovine sperm (32). However, the role of pH in sperm capacitation is not yet clear, since an artificial increase in intracellular pH in spermatozoa does not accelerate the process (16). Cross et al (33) have shown that cholesterol efflux from plasma membrane during capacitation of human spermatozoa determines a rise in intracellular pH and responsiveness to P. Recent data have demonstrated the presence of a Na+ dependent Cl-/HCO3- exchanger that regulates bicarbonate transport and motility in mature sperm (34), however the role of this pump in capacitation is not clear. It has been shown that bicarbonate regulates adenyl cyclase (AC) activity and cAMP metabolism (35) and is necessary for tyrosine phosphorylation of proteins during capacitation (see later). It is of interest that bicarbonate concentration is low in the epididymis and high in the seminal plasma and in the oviduct indicating that modifications of bicarbonate concentration in reproductive tracts play an important role in the suppression of capacitation in the epididymis and in the promotion of this process in the female reproductive tract (36).

2.2. Changes in membrane lipids and phospholipids during capacitation

Changes in the distribution and composition of plasma membrane lipids and phospholipids are another important feature of sperm capacitation. These changes lead to an increase in the membrane fluidity and to changes in the architecture and composition of plasma membrane (1). Cholesterol removal from the plasma membrane occurring during capacitation leads to a decrease in the cholesterol:phospholipid molar ratio in the sperm plasma membrane (reviewed in 37). It has been suggested that serum albumin present in the capacitating media may be responsible for removal of cholesterol (37). Visconti and coworkers (38) have demonstrated that heptasaccharides (cholesterol-binding molecules) promote the release of cholesterol from mouse sperm plasma membrane in absence of bovine serum albumin (BSA), increase tyrosine phosphorylation and promote capacitation of mouse sperm as measured by the ability of the ZP to induce the AR and by successful fertilization in vitro. These data suggest that cholesterol release is the signal that activates membrane signal transduction pathways related to capacitation (38).

The total amount of phospholipids does not appear to change considerably during capacitation (1). However, capacitation is associated with an increase of phospholipid methylation and increased synthesis of phosphatidylcholine from phosphatidylethanolamine (39). Incubation of spermatozoa in capacitating conditions, in the presence of bicarbonate, does not alter phospholipid distribution (40). Such condition, however, strongly inhibits phospholipid transfer and leads to a slow increase of phosphatidylcholine concentration in the inner leaflet of the membrane (40). Recently, Gadella and Harrison (41) have shown that inclusion of bicarbonate in the capacitating medium increases the translocation of phosphatidylcholine and sphingomyelin from the outer to the inner leaflet of the membrane, possibly due to activation of a bidirectional translocase (scramblase). Levels of phosphatidylinositol and lyso-phosphatidylcholine increase during capacitation in vivo in porcine sperm (42). In view of the fusogenic properties of lysophospholipids, an increase in their relative amount may be relevant to prepare the sperm for the acrosome reaction. Phospholipase A2 (PLA2) phospholipase C (PLC), and other phospholipases have been demonstrated in mammalian spermatozoa (43). The roles and activities of these enzymes in human sperm capacitation and acrosome reaction have been recently reviewed (43).

2.3. Changes in protein phosphorylation and protein kinase activity during capacitation

Protein phosphorylation during sperm capacitation has been widely studies in the last five years, leading to generation of more than 100 papers in the international literature. The best studied kinases involved in capacitation are Ca^{2+}-calmodulin activated kinases, cAMP-dependent kinases (PKA), calcium and phospholipid activated protein kinase (PKC), which induce phosphorylation of proteins in serine and threonine residues, and tyrosine kinases, which phosphorylate proteins in tyrosine residues. The involvement of kinase pathways in the process of capacitation has been mainly studied by using inhibitors of such pathways. However, specificity of kinases inhibitors is doubtful and many of them may affect other intracellular signal transduction pathways. In this light, caution should be applied in interpreting results of these studies.

2.3.1. Involvement of AC/cAMP/PKA pathway in capacitation

A spontaneous increase of cAMP during capacitation has been demonstrated (44) and inhibitors of protein kinase A, the serine-threonine kinase activated by this pathway, inhibit capacitation (45). It has been well established that Ca^{2+} and HCO3- stimulate adenylyl cyclase (AC) but the exact mechanism by which these ions activate AC is not clear (35,46). cAMP generated during capacitation and subsequent activation of PKA appears to play a key role in the increase of tyrosine phosphorylation during capacitation (47, see also later). Since PKA may phosphorylate several cellular substrates, its sequestration in specific cellular compartments is necessary to spatially restrict its action, thus ensuring specificity of functions. Compartmentalization of PKA is accomplished by A kinase-anchoring proteins (AKAPs) which have been recently characterized in sperm of different species (48-50). Their specific localization to the tail indicate a role for these proteins in the modulation of sperm motility (50). Pentoxifylline, which promotes an increase in cAMP by inhibiting sperm phosphodiestarases, induces capacitation (51). Recently, a role for AC/cAMP/PKA pathway in the plasma membrane lipid remodelling occurring during capacitation has been also demonstrated (52). Many questions remain still open concerning the AC/cAMP/PKA pathway in human sperm. Indeed, although recently the specific expression of a soluble adenylate cyclase has been shown in male germ cells (53), the types of phosphodiesterases (PDEs) present in these cells remain to be defined. Recently, Richter et al (54) detected, by RT-PCR, the presence of mRNA transcripts for several PDEs in ejaculated human spermatozoa, but no conclusions can be drawn concerning the type of PDE specifically expressed in mature spermatozoa.

2.3.2. Involvement of PKC in capacitation

The presence of PKC in mammalian spermatozoa and its role in sperm motility and the process of acrosome reaction are documented (55), but PKC activity is very low compared to somatic cells (55,56). The role of this enzyme during capacitation is poorly understood. Early studies demonstrated that stimulation of PKC with phorbol esters accelerates the process of capacitation (57). This effect was inhibited by PKC inhibitors, suggesting that PKC may be involved in capacitation (57). In addition, PKC may also be involved in epidermal growth factor- induced capacitation (58). All these studies were performed using high levels of phorbol esters as PKC inducers. Although we have shown that PKC activity of human sperm can be stimulated by a phorbol ester (56), additional effects of these tumor promoters on other sperm kinases cannot be excluded. Recently, the role of PKC in capacitation has been questioned (51).

2.3.3. Involvement of tyrosine phosphorylation in capacitation

The first evidence for the presence of tyrosine phosphorylated proteins in mammalian spermatozoa dates back to 1989 (59). Using anti-phosphotyrosine antibodies, Leyton and Saling identified three different phosphoproteins at 52, 75, and 95 kDa in the mouse spermatozoa (59). The 95 kDa protein was tyrosine phosphorylated under all experimental conditions and including interaction of spermatozoa with solubilized ZP proteins (59). Capacitation is characterized by a spontaneous, time-dependent increase of tyrosine phosphorylation of different proteins (60,61). The main tyrosine phosphorylated proteins are in the range of 95-100 kDa (59-63). At least in the mouse, the increase of protein tyrosine phosphorylation is dependent on the presence of Ca^{2+}, NaHCO3- and BSA (61). It is worth to note that some of these results have not been confirmed in human sperm. Indeed, in this specie Ca^{2+} and tyrosine phosphorylation seem to be inversely related and protein tyrosine phosphorylation is enhanced in calcium free medium (62,48) indicating that calcium might induce the activation of tyrosine phosphatases (48). Interrelationships between BSA and cholesterol removal appear to be also important in protein tyrosine phosphorylation. It has been indeed demonstrated that in BSA-deprived media protein tyrosine phosphorylation and sperm capacitation are inhibited (61,63).

As mentioned above, during capacitation tyrosine phosphorylation is regulated by AC/cAMP/PKA pathway (63,64) as well as by reactive oxygen species generated at the beginning of capacitation (65, see later). In addition, recent data suggest a role for nitric oxide generated by human spermatozoa in the promotion of capacitation (66,67) and the increase of tyrosine phosphorylation (66). As stated above, in human sperm the highest degree of tyrosine phosphorylation was found in a protein of 95-97 kDa (59-63). A tyrosine phosphorylated protein in this molecular weight range was previously indicated as the possible sperm receptor for ZP3, ZRK (68). This protein has been characterized, partially cloned and sequenced. Its sequence shows a 55% homology with the receptor-like protein tyrosine kinase c-eyk (68) and 97-100% homology with the proto-oncogene c-mer (69). Among the other tyrosine phosphorylated proteins in the same molecular weight range, it has been recently identified an A-Kinase

Anchor Protein (AKAP) specifically expressed at the tail level (48-50) which may be involved in the

Table 1. Molecules that can induce acrosome reaction *in vivo*.

AGONIST	REFERENCE
Zona pellucida protein (ZP3)	1, 80
Serum albumin	1
Epidermal growth factor	58
Atrial natriuretic peptide	55
Platelet-activating factor	85,105,106
Progesterone & 17βOH-progesterone	11,81
ATP	84
Prostaglandin E1	115

development of hyperactivated motility pattern. In addition, we have identified extracellular signal regulated kinases (ERK-1 and ERK-2) as two sperm proteins that are phosphorylated in tyrosine and activated during sperm capacitation (70,71). Their inhibition with a pharmacological compound suppresses capacitation (70), indicating a role of these proteins in the process. Immunofluorescence labelling of phosphotyrosine residues, indicated that capacitation as well as exposure to zona proteins increased the degree of tyrosine phosphorylation in each spermatozoon and the number of sperm cells phosphorylated in the acrosomal region of the sperm head (72). Incubation of spermatozoa with antiphosphotyrosine antibodies or inhibition of tyrosine kinase activity inhibited zona-free hamster egg penetration (72), prevented acrosome reaction and blocked fertilization (73). However, whether tyrosine phosphorylation is fundamental for the development of the capacitated state or is simply associated to the phenomenon is matter of debate. We have shown that erbstatin, a potent inhibitor of tyrosine kinase, did not inhibit capacitation measured as the ability of spermatozoa to respond to P (62). Similarly, Ain et al (51), have recently reported that thyrphostin A-47, a protein tyrosine kinase inhibitor, does not inhibit pentoxifylline-stimulated capacitation, although suppressed acrosome reaction stimulated by this agent. To understand the role of tyrosine phosphorylation in the process of capacitation, we will probably need to wait for the characterization of many, if not all, of the tyrosine phosphorylated proteins as well as the tyrosine kinases that are activated during the process. Presence of tyrosine kinases, including c-ras (74), EGF-receptor tyrosine kinase (75), the cell-cycle specific cyclin, cdc-2 (76), and p190 c-met tyrosine kinase (77) have been demonstrated in mammalian spermatozoa but their role in capacitation is not yet clear.

3. THE ACROSOME REACTION (AR): BACKGROUND

AR is an exocytotic process physiologically induced by ligand (ZP3)-receptor interaction, consisting in multiple fusions between outer acrosomal membrane and the overlaying plasmamembrane leading to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface that mediate fusion with oolemma. The sperm acrosome is a Golgi-derived structure forming a cap over the anterior region of the nucleus which contains many hydrolytic enzymes and consists of an anterior cap and a posterior region called equatorial segment (1). As mentioned above, only capacitated sperm are physiologically able to undergo AR in response to physiological stimuli. It is thus conceivable that the two process, capacitation and AR, are sequentially and functionally linked such that several of the effectors involved in mediating intracellular signaling activated by AR start to be tuned during capacitation. For instance, the increase of intracellular calcium levels and tyrosine phosphorylation of proteins accompanying capacitation is also essential for the subsequent exocytosis in response to P (13,78). The process of AR consists of development of multiple fenestrations between the outer acrosomal membrane and the plasma membrane of the spermatozoa (1) leading to the release of the enzymatic content of acrosome and to the exposure of the enzymes bound to the inner membrane adjacent to the nuclear envelope (1). In the absence of any specific stimuli only a low percentage of human spermatozoa can undergo AR (79). It has been suggested that self aggregation of the sperm receptor for ZP may account for this spontaneous acrosome reaction (80). A wide variety of molecules present on sperm surface have been proposed as putative candidate for ZP3 receptor (36). This rather long list of possible candidates may be justified by the involvement of different receptors in different species or different binding affinities, or even multiple receptors that may cooperate in sequence to induce AR: however none of these molecules has been definitively recognized as “the sperm-egg receptor” (36). AR can also be physiologically induced by P (P), present at high levels in the cumulus matrix surrounding the oocyte that must be crossed by sperm to reach the zona pellucida. The steroid has been described to affect several other sperm functions, including capacitation, motility and priming effect on ZP3-induced AR through stimulation of a rapid nongenomic signaling pathway mediated by recently characterized P receptors present on sperm surface (for further details on effects of P see 11 and 81). P-induced AR is inhibited by 17β-estradiol through interaction with a specific nongenomic estrogen receptor on sperm plasma membrane (82), suggesting that estradiol, which is present at micromolar levels in the follicular fluid, may act as a physiological modulator of P action on sperm assuring the appropriate timing of activation in the fertilization process. Other agonists proposed to act as in vivo inducers of AR are listed in table 1.

Many molecular mechanisms have been demonstrated to be active during AR (Figure 2), but for many of them a precise cause-effect relationship has not yet been defined, such that we still do not know whether they are essential for the process of fertilization or they are simply associated with the process. The fundamental difference between P- and ZP3-induced AR stands on the nature of their receptors. In fact, in the case of ZP, its action is mediated by receptor recruitment of G proteins which are not involved in P stimulated cascade (36). One of the first events that occurs in spermatozoa following stimulation with ZP3 and P is receptor aggregation (80,83). This is followed by a cascade of downstream membrane and cytosolic signaling factors involved in induction of AR (schematized Figure 2). Among them, the roles of calcium, phospholipases and protein kinases are discussed below.

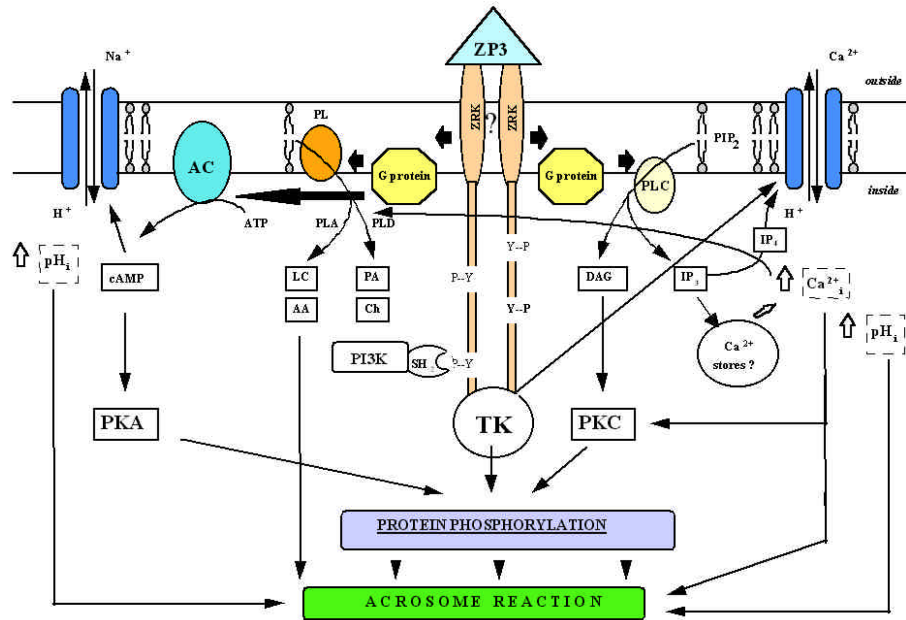

Figure 2. Diagram illustrating the main signal transduction pathways activated during the process of acrosome reaction in response to zona protein 3 (ZP3). Following interaction with the agonist, aggregation of receptors for ZP3 (ZRK ?) induces TK activation (which increases protein tyrosine phosphorylation) and autophosphorylation of the receptor. Activation of phosphatidylinositol-3kinase (PI3K) has been also reported. A guanine nucleotide binding protein (G protein) transduces the signal interacting with membrane-bound enzymes like phospholipase C (PLC) and adenylate cyclase (AC). Activation of these two enzymes leads to increased generation of the second messengers cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP3) and diacylglycerol (DAG). A consequence of the increase of second messengers is the activation of protein kinases such as cAMP-dependent kinase (PKA) and Ca^{2+} and phospholipid-dependent kinase (PKC) with increased protein phosphorylation. cAMP-dependent influx of sodium (Na+) has been reported. IP3 may increase intracellular Ca^{2+} by liberation of the ion from intracellular Ca^{2+} stores. The increase of intracellular Ca^{2+} consequent to activation of ZP3 receptors is completely due to influx from the extracellular medium, is dependent on activation of G proteins, involves voltage-dependent Ca^{2+} channels and is accompanied by an efflux of H+ which determines a rise of intracellular pH (pHi). Partial dependence of Ca^{2+} -influx from TK activation has been reported. Ca^{2+} dependent activation of phospholipase A 2 (PLA2) and phospholipase D (PLD) [with increased generation of other second messengers as arachidonic acid (AA), lyso-phosphatidylcholine (LC) and phosphatidic acid (PA) from membrane phospholipids (PL)] have also been described to occur during acrosome reaction.

3.1. Increase in intracellular calcium during acrosome reaction

Calcium plays a central role in receptor-mediated response and membrane fusion processes in spermatozoa (1,36). Calcium ionophores are indeed the most widely used non physiological inducers of AR (1). Moreover, although AR can be induced in the absence of extracellular calcium with some agonists (84-86) stimulation of calcium fluxes is one of the earlier responses activated by most stimuli that induce AR. Interestingly, the shape of calcium wave is different in P- and ZP- stimulation. While for P a biphasic calcium wave is described, consisting in a rapid initial peak followed by a long lasting plateau phase (11), ZP3 induces a slow and sustained increase of calcium (10), resembling the second phase of P. However, it has been recently shown, using a different methodological approach, that ZP3 may induce a rapid increase of intracellular calcium concentrations occurring within milliseconds after stimulation (87). Parallel inhibition of both AR and the second phase of calcium response to P by both tyrosine kinase blockers and a previous administration of estradiol, suggests the plateau phase to be responsible for agonist-induced AR (88,89,81). Recently, a direct relationship between the sustained calcium phase and AR in response to ZP3 (20) has been demonstrated. Concerning P, such a relationship is less apparent, since the percentage of sperm undergoing AR in response to the steroid is smaller than that where a sustained response is observed (21,22). Although extracellular calcium depletion totally prevents both ZP and P-induced intracellular calcium increase, recent data suggest the possible involvement of intracellular calcium stores in sperm AR (18-20, see also above). In particular, it has been recently shown that calcium entry during the sustained phase in response to ZP3 is due to activation of store-operated channels (20). While membrane voltage-operated calcium channels of T-type have been demonstrated in mediating ZP-induced calcium influx (24,25), the nature of P-stimulated calcium channels is still a matter of discussion (14,90,91). Calcium plays a key role in the fusion events in the sperm membrane (92). Using a pyroantimonate-osmium fixation technique, the temporal and spatial location of intracellular calcium granules was monitored during acrosome reaction

in ram spermatozoa (92). Ca^{2+} is initially associated with the outer acrosomal membrane. As the process progresses, Ca^{2+} associates with the fusion sites between the outer acrosomal membrane and the plasma membrane anteriorly to the equatorial segment. At later stages, Ca^{2+} is localized in both post acrosomal dense lamina and on outer acrosomal membrane under the equatorial segment. These findings suggest that Ca^{2+} may be implicated in the fusion process (92).

Increase of intracellular Ca^{2+} in response to ZP3 and P is associated with an efflux of H+ and a rise in intracellular pH (93-95). Moreover, P has been shown to rapidly stimulate sodium influx (91) and the presence of sodium in the extracellular medium is absolutely required for induction of AR by the steroid (91,94). Sperm intracellular pH may be regulated by a Na+/H+ exchanger (96) as well as by Cl-/HCO3- (97), whose presence have been demonstrated in germ cells. For further details in ion channels in spermatozoa see Darszon et al (25).

3.2. Phospholipases activation during acrosome reaction

It was shown earlier that during AR in boar spermatozoa, the amount of diacylglycerol and free fatty acids increases (98). This finding was consistent with activation of phospholipases (43). In fact, increase of $[Ca^{2+}]i$ stimulated by ionophores and P leads to activation of phospholipase C in human spermatozoa (43). Such activation leads to an intracellular increase in the amount of inositol trisphosphate and diacylglycerol. Similarly, ZP3 activates the phosphoinositide specific enzyme phospholipase Cγ1 (99) by virtue of tyrosine phosphorylation and leads to its translocation from cytosol to particulate fractions (99). Presence and activation of phosphatidylcholine-specific phospholipase C during AR has been also demonstrated (43). Diacylglycerol (DAG) and inositol-trisphosphate (IP3), produced by calcium-dependent activation of PLC, may be respectively involved in the regulation of PKC activity and release of calcium from putative sperm intracellular stores (18,55).

AR induced by ionophores and P also leads to the activation of phospholipase A2 (43). This activation is associated with generation of lipid metabolites, such as arachidonic acid and lysophospholipids. Phosphatidylcholine, lysophospholipids, and unsaturated fatty acids, such as arachidonic acid, are potent inducers of AR (100-102) and may be implicated in the fusion process that occurs during AR. Moreover, lysophosphatidylcholine generated from PLA2 activation, may act as a substrate for generation of platelet-activating factor (103,104). This phospholipid, that is synthesized in response to P (103), may further enhance AR (85, 105,106). Moreover, in vitro treatment of human sperm with platelet activating factor (PAF: 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) enhances penetration of zona free hamster oocytes (107). In animal studies, in vitro treatment of sperm with PAF significantly improves fertilization rate using both ICSI and IVF techniques without showing detrimental effects on subsequent embryo development (108,109).

3.3. Involvement of protein kinases in acrosome reaction process

During AR, activation of protein kinases (serine-threonine kinases as PKC, PKA and PKG, and tyrosine kinases) is downstream to the production and/or activation of early second messengers. Early evidence for the involvement of PKA in the AR, demonstrated that adenylate cyclase activity and cAMP generation increase during this process (110). Adenylcyclase stimulators, such as forskolin, and the cAMP analogue dibutyryl cAMP, induce AR in a dose-dependent manner in mammalian sperm (22, 111-113). In addition, PKA inhibitors have been reported to suppress acrosome reaction induced by P (113), although controversial results are present in the literature concerning an increase of cAMP synthesis in response to P (114,115). Taken together, this findings suggest that PKA activation may be involved in AR process.

The involvement of PKC in the process of AR is still discussed. Indeed, although activators such as phorbol esters and synthetic DAG, induce acrosome reaction (55), PKC activity in humans sperm is, as mentioned above, very low. Moreover, the identity of PCK substrates is still under investigation. Induction of AR by solubilized zona pellucida was partially reduced by pretreatment with inhibitors of PKA, PKC and PKG tested separately, while combination of them caused a significantly greater inhibition (86). These results suggest a concomitant role for PKA, PKC and PKG in human ZP-induced acrosome reaction (86). Both P (56,116) and ZP (117) increase sperm PKC activity. Such activation is downstream to calcium influx (56), since it can be prevented by calcium channel blockers (116).

ZP3 induced an increase in tyrosine phosphorylation of sperm proteins (59,68). A major tyrosine phosphorylated protein in mammalian spermatozoa was 95-97 kDa (59,68). Proteins in this molecular weight range appear to undergo phosphorylation during capacitation (see above) and in response to P (11). Recombinant human ZP3 produced by genetic engineering, has been demonstrated to induce AR and tyrosine phosphorylation of the 95-110 kDa protein in human sperm (118), perfectly mimicking the physiological action of this glycoprotein. Known inhibitors of tyrosine kinase, such as genistein and Tyrphostin 47, block ZP3-induced AR (73). Moreover, both the tyrosine kinase inhibitor tyrphostin A48 and pertussis toxin suppress ZP3-induced calcium influx in mouse spermatozoa (119). Using a similar pharmacological approach, Tesarik et al. (120) and Luconi et al. (60) showed involvement of tyrosine kinases in the P-mediated acrosome reaction. Activation of tyrosine kinase seems also to be involved in the plateau phase of increase in the amount of intracellular Ca^{2+} in response to P (88,89). The mechanisms through which tyrosine kinases are activated in sperm during the process of acrosome reaction are still under investigation. Although during capacitation activation of Adenylate cyclase/cAMP/PKA pathway appears to be highly involved (see above) activation of this pathway in response to stimuli that induce AR is less clear (114,115). Recently, the involvement of reactive oxygen

species generated by sperm in response to stimuli that induce AR, in tyrosine phosphorylation has been suggested (121,122) .

Recent data (123) indicate the involvement of phosphatidylinositol-3kinase (PI3K) in acrosome reaction stimulated by agents that mimic ZP3, such as mannose-BSA and polyclonal antibodies raised against the p95 protein indicated as the possible sperm ZP3 receptor (ZRK, see above), but not by P or ionophores.

4. DNA FRAGMENTATION IN HUMAN SPERMATOZOA

It is well known that in human ejaculated sperm there is a variable percentage of DNA fragmented cells, as detected by several techniques including TUNEL (124-126), in situ nick translation (127) and COMET (127,128). Up to now, the origin of this kind of DNA damage and, most important, the fertilizing ability and the consequences on the embryo development of a damaged spermatozoon are not thoroughly known. The finding that DNA fragmentation is correlated to poor sperm parameters (124-127) has increased the concern over the patients being treated by in vitro fertilization technologies, in particular by ICSI . Indeed, the mechanical bypass of the natural barriers of the oocyte during the ICSI, may increase the likelihood that a damaged spermatozoon will participate in fertilization and embryo development. Hence, in the last decade the awareness of the importance of studying and detecting sperm DNA integrity has greatly increased.

4.1. Origin of sperm DNA damage

Several theories have been proposed to explain the occurrence of DNA fragmentation in human ejaculated spermatozoa. The first theory stems from studies in animal models. Mc Pherson and Longo (129) observed that DNA breaks occur and later on disappear, during the spermiogenesis. The authors showed also that topoisomerase II might be involved in the re-ligation of the DNA nicks (130) and proposed that the transient occurrence of sperm DNA breaks might have a favouring role in the histone-to-protamine substitution (129). In this light, the DNA fragmented spermatozoa could be interpreted as cells that failed to complete maturation and in particular to complete package of chromatin. Some findings support this speculation: i) a close direct correlation has been reported between DNA breakage and both poorly protaminated chromatin (131) and increased sensitivity of DNA to denaturation (an index of less stable and resistant chromatin) (132) and ii) DNA fragmented sperm also display persistent cytoplasmic residues, as detected by electron microscopy (126).

Another theory states that DNA breakage could be an index of programmed cell death (i.e. apoptosis), as occurring in somatic cells (133). Apoptosis has been extensively observed in human and animal testis (134-136). It is believed that testicular apoptosis has the important function to match the number of germ cells with the amount of the supportive Sertoli cells (14). Exceeding germ cells would be deleted via the interaction between Fas receptor (on their surface) and Fas ligand (expressed by Sertoli cells) (136,138). Moreover, many studies show that apoptosis in seminiferous epithelium is controlled by hormones, in particular FSH (134,135).

The standard tools used to reveal apoptosis are unable to verify the occurrence of classical hallmarks of the phenomenon in mature ejaculated sperm. Searching for laddering of nucleosomes is not suitable in cells that have nearly completely lost the nucleosomal arrangement. Furthermore, TUNEL labelling cannot be considered itself indicative of apoptotic DNA cleavage (131), which is at variance with somatic cells. Nevertheless, ultrastructural features resembling somatic apoptosis (139-141) have been described in ejaculates with poor sperm morphology (139,140). It has also been demonstrated that such apoptotic-like submicroscopic sperm characteristic are reverted by a short term treatment with FSH (140). In addition, Sakkas et al reported an increased amount of Fas-positive cells in mature sperm of oligozoospermic men (137). Finally, binding of Annexin V has been observed in ejaculated spermatozoa simultaneously assessed for the viability (i.e. exclusion of the DNA stain propidium iodide) and thus interpretable as apoptotic sperm (142).

The association between abnormal semen parameters and both ultrastructural apoptotic signs and sperm Fas expression, induced Sakkas et al (137) to postulate the occurrence of an “abortive apoptosis”. Abortive apoptosis is a process that begins in the testis and fails to complete because of impairment in the program of cell death or mismatching with spermatogenesis. Thus death committed cells can not be completely deleted and can be observed in the semen altogether with other abnormal sperm parameters. Sakkas et al also speculates (14) that abortive apoptosis is not in contrast with the theory that the endogenous DNA breakage is indicative of a failure in the chromatin packaging. In fact triggering of apoptotic program by committed spermatides could be the actual cause of the failure in the nuclease activity creating and ligating nicks during spermiogenesis (129,130). However, annexin V staining is indicative of the translocation of the membrane phosphatidylserine, thus of the occurrence of a very early stage of apoptosis. Indeed, occurrence of annexin V labelled ejaculated sperm does not exactly match with a cell death program triggered in the testis. In addition, the amount of DNA fragmentation does not correlate with the ultrastructural and FSH revertible apoptotic-like sperm features (126).

Another proposed cause of DNA strand breakage is the oxidative stress. A large variety of semen factors are known to generate ROS, including spermatozoa themselves (121,122). In addition, both endogenous (143) and exogenous (144) free radicals are known to attack sperm DNA. High level of ROS in seminal plasma have been associated with poor sperm parameters (145,146) and infertile patients show increased amount of 8-hydroxydeoxyguanosine (a biomarker of oxidative DNA damage) (147). Moreover, a positive correlation has been reported between sperm DNA breakage and sperm ROS generation (142). Even if ROS can directly produce DNA strand breaks, they could cause sperm DNA breakage also

by triggering an apoptotic process, as occurs in somatic cells (148). If so, apoptosis could stem also from post-testicular sites, i.e. in the genital tracts where sperm transit and are stored (142).

In conclusion, the origin of sperm DNA breaks is not yet defined. In particular it is not clear whether DNA fragmentation can be considered an executive step of a program of cell death. Provided that apoptosis occurs , further investigation is necessary to understand the sites, the time and the mechanism underlying the phenomenon.

5. PERSPECTIVE

Studying in vitro capacitation has allowed characterization of a number of biochemical events that occur in human spermatozoa. It is not clear whether the same or similar events occur during capacitation in vivo. P, platelet-activating factor and other effectors present in the follicular fluid and/or cumulus matrix, could facilitate capacitation or prime the acrosome reaction in vivo. For instance, the clinical significance of P effects has been shown by several studies showing a significant correlation of response to P with sperm fertilizing ability as well as reduced or absent response to P in infertile subjects (11,81). Moreover, treatment of human sperm with P has been shown to enhance hamster oocyte penetration (149). These studies indicate that sperm P receptor, as well as the one that mediates ZP3 stimulation, is involved in the process of fertilization representing a possible target for developing pharmacological strategies to potentiate sperm fertilizing ability in assisted reproductive techniques or contraceptive molecules. Functional P receptors on human sperm surface have been recently characterized (150,151), sequencing of the receptor and cloning of encoding gene will represent next steps in such characterization.

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