[Frontiers in Bioscience 1, d189-205, August 1, 1996]

HUMAN SPERM ACTIVATION DURING CAPACITATION AND ACROSOME REACTION: ROLE OF CALCIUM, PROTEIN PHOSPHORYLATION AND LIPID REMODELLING PATHWAYS.

Elisabetta Baldi[1], Michaela Luconi, Lorella Bonaccorsi, Csilla Krausz and Gianni Forti.

Dipartimento di Fisiopatologia Clinica, Unita' di Andrologia, Universita' di Firenze, viale Pieraccini 6, I-50139 Firenze, Italy.

TABLE OF CONTENTS

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 property of spermatozoa render these cells 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 exposes 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 are only partially defined, and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodelling in sperm plasma membrane as well as changes in protein phosphorylation. The human and mouse sperm receptor for ZP3 has been recently sequenced and cloned. This receptor exhibits sequence homology with proto-oncogenes that mediate proliferation and differentiation in somatic cells. This review summarizes the main signal transduction pathways involved in capacitation and acrosome reaction.

Received 5/30/96; Accepted 7/30/96

[1] To whom correspondence should be addressed, at Dipartimento di Fisiopatologia Clinica, Unita' di Andrologia, Universita' di Firenze, viale Pieraccini 6 I-50139 Firenze-Italy. Tel #: +39.55.4221816; Fax #: +39.55.4221848 Email: e.baldi@mednuc2.dfc.it

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. These fundamental processes normally take place in the female genital tract during the migration of spermatozoa to the site of fertilization (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. One of the functional consequence of capacitation is development of a distinct motility pattern called hyperactivation, which is characterized by pronounced flagellar movements, marked lateral excursion of the sperm head and a non linear trajectory. 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

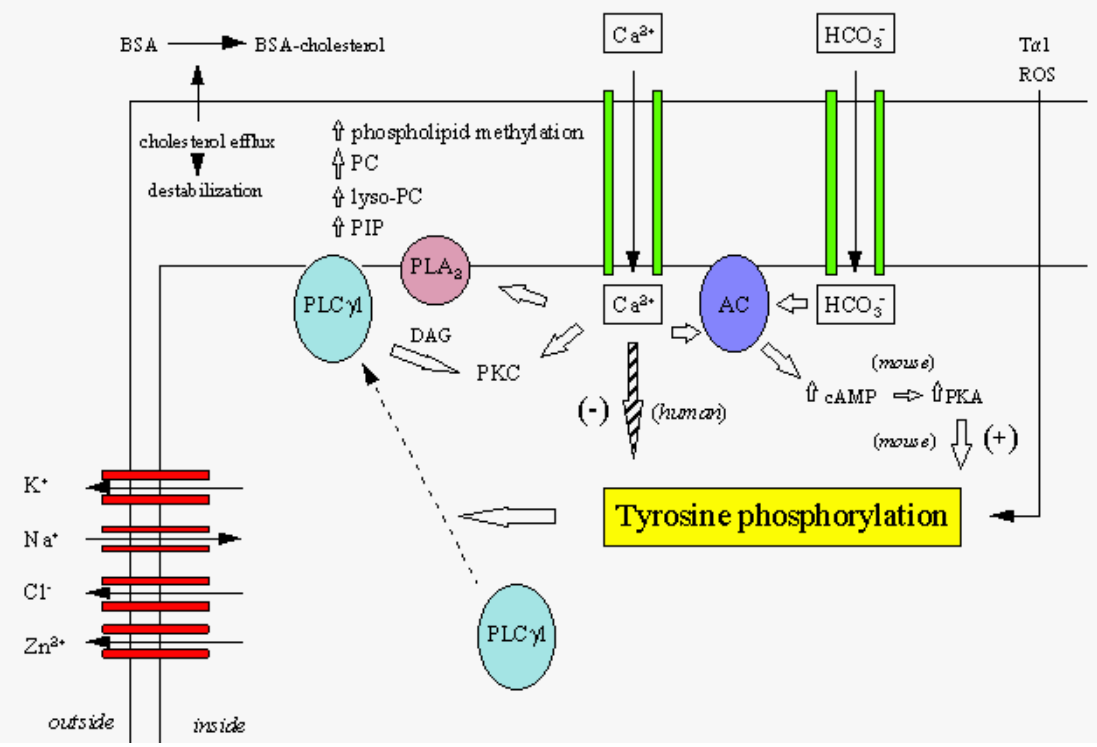

Figure 1. Schematic representation of the main events occurring under conditions leading to capacitation *in vitro*. Changes in membrane permeability to several ions have been described, among these Ca^{2+} and bicarbonate (HCO_3^-), whose influx increase during capacitation, have been reported to exert a primary role in the process. Membrane fluidity increases due to the loss of cholesterol from the membrane which may be accelerated by the extracellular presence of serum albumin (BSA). A time-dependent increase of tyrosine phosphorylation of proteins has also been described. In mouse sperm, the increase of tyrosine phosphorylation is primarily dependent on the increase in Ca^{2+} and bicarbonate, which, in turn, activate 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). On the contrary, in human sperm Ca^{2+} inhibits, rather than increases, tyrosine phosphorylation during capacitation, indicating the existence of different regulatory pathways of this process among different species. Other possible physiological modulators of tyrosine phosphorylation in human sperm during capacitation are Tα1 (present in seminal plasma and in oviductal fluids) and reactive oxygen species (ROS) that may generate from spermatozoa. Remodelling of sperm membrane phospholipids and activation of phospholipases (PLA2 and PLCγ1) have also been shown: in particular, increased synthesis of phosphatidylcholine (PC) from phosphatidyl-ethanolamine, phosphatidylinositol (PIP) and lyso-phosphatidylcholine (lyso-PC) have been documented. In the mouse a translocation of PLCγ1 from a soluble to a particulate fraction during capacitation has been demonstrated. A possible activation of protein kinase C (PKC) has also been reported. ⇒, stimulatory pathway; ⇒, inhibitory pathway.

protein ZP3 and progesterone (1). The responsiveness of spermatozoa to ZP3 (1-2) and to progesterone (3-5) increases during capacitation, assuring maximal responsiveness at the site of fertilization. Capacitation is associated with modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activities and modulation of expression of intracellular constituents (1). However the exact relationship among these modifications is not yet completely understood. In addition, there is as yet no clear-cut method that allows distinction of capacitated from noncapacitated spermatozoa (6). Capacitation does not occur synchronously in spermatozoa (7). In addition, capacitation is transient and already capacitated spermatozoa cannot be capacitated again (8). 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(Fig1).

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

Modification of intracellular concentration of calcium ions (Ca^{2+}) 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 (9-14) including human (3,15). Extracellular Ca^{2+} appears to be necessary for the completion of capacitation of spermatozoa *in vitro* (15-20) and the increase of intracellular Ca^{2+} is required for capacitation (19-20). In spermatozoa the intracellular Ca^{2+} is regulated by the Ca^{2+}-ATPase (acting as a Ca^{2+} extrusion pump) (21), Ca^{2+}/H^{+} exchanger system plus Na^{+}/Ca^{2+} antiporter (acting as Ca^{2+} entrance systems) in the plasma membrane (22), and possibly, by the intracellular Ca^{2+} stores (23-24). It has been hypothesized that modulation of the activity of the Ca^{2+}-extrusion system, in particular Ca^{2+}-ATPase, occurs during capacitation and leads to an increase in intracellular Ca^{2+}(21-25). Drugs such as quercetin, that inhibit Ca^{2+}-ATPase accelerate capacitation (21-22,25-27) whereas calmodulin inhibitors such as trifluoperazine and naphtalensulfonamide (W-7) enhance capacitation (21-22,25-27). A Na^{+}/Ca^{2+} exchanger is present in mammalian sperm, however, its role in controlling intracellular Ca^{2+} during capacitation is not clear. A low molecular weight protein, caltrin, associated with ejaculated bull spermatozoa, which inhibits the Na^{+}/Ca^{2+} exchanger maintains the intracellular Ca^{2+} at low levels (27). In the female genital tract, conformational changes appear to allow this protein to stimulate the exchanger and induce a Ca^{2+} in/Na^{+} out movement (27). Ca^{2+} channels have been demonstrated in mammalian sperm (2, 28-29), however, their role in modulating sperm intracellular Ca^{2+} during capacitation is controversial (22). The increase in intracellular Ca^{2+} during capacitation is a slow process reaching a plateau after 90-120 minutes from the beginning of the process (3). This is inconsistent with the mode of action of Ca^{2+} channels that usually mediate a rapid influx of Ca^{2+} suggesting that these channels are involved in the influx of Ca^{2+} preceding the acrosome reaction (22).

Besides Ca^{2+}, intracellular K^{+} (30), Na^{+} (31) and Cl^{-} (22) concentrations have been shown to be modulated during capacitation. Moreover, Na^{+},K^{+}-ATPase activity increases in the hamster spermatozoa during this process (27). Activation of this pump is expected to be associated with a decrease in the intracellular concentration of Na^{+}. However, paradoxically, increase in the intracellular concentration of this ion has been reported (22, 31). Such increase of intracellular Na^{+} appears to be important for capacitation, since the Na^{+} ionophore, monesin, promotes this process in mouse sperm (22).

A rise in intracellular pH has been reported during capacitation of bovine (32) but not hamster (10) sperm. However, the role of pH is not apparent since the increase in pH in spermatozoa does not accelerate capacitation (22). On the other hand, an important role for bicarbonate in the capacitation has been shown in several mammalian species (33-36,19). The action of bicarbonate may be related to its role in stimulation of adenylate cyclase activity rather than pH buffering capacity (37-38). The intracellular concentration in zinc ion decreases in the acrosome of hamster spermatozoa during capacitation (39). In addition, incubation of spermatozoa in a zinc-containing medium inhibits capacitation (39). These findings suggest that zinc may play a role in destabilization of plasma membrane during capacitation (39).

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 (1). Among these changes is cholesterol removal which leads to a decrease in the cholesterol:phospholipid molar ratio in the sperm plasma membrane (40-46). It has been suggested that serum albumin may be responsible for the cholesterol removal (46-48). However, such an effect may simply be induced by washing of the spermatozoa (49). Purified lipid transfer protein, a serum protein present in the follicular fluid which is able to increase sperm capacitation (50), may provide the physiological mechanism for *in vivo* capacitation.

The 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 phosphatidyl-ethanolamine (51). Incubation of spermatozoa in capacitating conditions, in the presence of bicarbonate, does not alter phospholipid distribution (52). Such condition, however, strongly inhibits phospholipid transfer, but leads to a slow increase of phosphatidylcholine concentration in the inner leaflet of the membrane (52). Levels of phosphatidylinositol and lyso-phosphatidylcholine increase during capacitation *in vivo* in porcine sperm (53). In view of the fusogenic property of lysophospholipids, increase in their relative amount may prepare the sperm for the acrosome reaction. Phospholipase A2 is an enzyme that generates lysophospholipids through hydrolysis of phospholipids (54). This enzyme seems to be implicated in sperm capacitation (55). Moreover, the presence of another enzyme, phospholipase Cγ1 (PLCγ1), that leads to hydrolysis of phosphoinositides has been demonstrated in mouse spermatozoa (56). During capacitation mouse

spermatozoa PLCγ1 translocates from a soluble to a particulate fraction (56). This translocation may be required for establishment of contact between this enzyme and activated membrane protein tyrosine kinases in the mammalian spermatozoa (see later). In addition, capacitation is associated with a shift in the electrophoretic mobility of PLCγ1 (56). Such slower electrophoretic mobility may be due to tyrosine phosphorylation and activation of PLCγ1 (57-58).

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

Generally, protein phosphorylation is stimulated by activation of kinases (59). 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.

2.3.1. Involvement of PKA in capacitation

Adenylcyclase activity and synthesis of cAMP increase during capacitation of mouse sperm (60-62). cAMP agonists and phosphodiesterase inhibitors counteract the inhibitory effect of glucose on capacitation of bovine spermatozoa (63). Pentoxifylline, which promotes an increase in cAMP, induces capacitation (64). These findings suggest that PKA may be involved in the process of capacitation. A more direct evidence for the involvement of cAMP-dependent kinases in the process of capacitation has been recently reported (38). Two different inhibitors of PKA inhibited capacitation (38). Moreover, the process of capacitation could be induced by addition of biologically active cAMP agonists (38). The increase in concentration of cAMP in spermatozoa is dependent on presence of Ca^{2+} and bicarbonate in the extracellular medium (65). Inhibitors of PKC and Ca^{2+}/calmodulin dependent protein kinases, however, do not seem to have any effect on the concentration of cAMP (38).

2.3.2. Involvement of PKC in capacitation

Presence of PKC in mammalian spermatozoa and its role in sperm motility and the process of acrosome reaction are documented (66-68). However, the role of this enzyme during capacitation is poorly understood. Stimulation of PKC with phorbol esters accelerates the process of capacitation (69). This effect was inhibited by PKC inhibitors, suggesting that PKC may be involved in capacitation (69). In addition, PKC may also be involved in epidermal growth factor- induced capacitation (70). However, these studies were performed using phorbol esters as PKC inducers, and non specific effects of these tumor promoters on other kinases cannot be excluded. PMA and diacylglycerol stimulate the acrosome reaction only in spermatozoa that have been previously capacitated (68,71). Such evidence favours the concept that PKC pathway is implicated in capacitation.

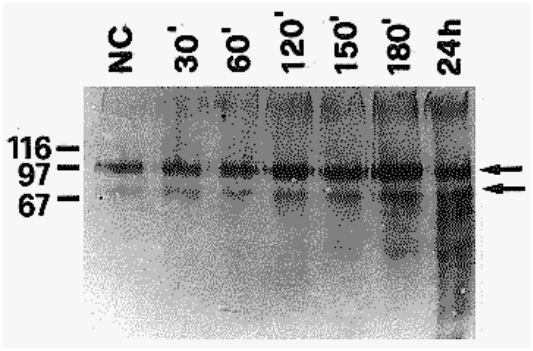

Figure 2. Western blot analysis of the reactivity of the anti-phosphotyrosine antibody with human sperm proteins at various times of *in vitro* capacitation. NC indicates capacitation at 0 min. Tyrosine phosphorylated proteins were revealed using an anti-phosphotyrosine polyclonal antibody followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody and stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. Molecular weight markers (kDa) are indicated to the left of the blot. The arrows indicate the 97 and the 75 kDa phosphoproteins. From Luconi *et al*. (74) with permission.

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 (72). Using anti-phosphotyrosine antibodies, Leyton and Saling identified three different phosphoproteins at 52, 75, and 95 kDa in the mouse spermatozoa (72). The 95 kDa protein was tyrosine phosphorylated under all experimental conditions and including interaction of spermatozoa with solubilized ZP proteins (72). The 75 kDa and 52 kDa proteins were phosphorylated only in capacitated spermatozoa and may represent capacitation-specific markers (72). Later on, similar tyrosine phosphorylation pattern was reported in human spermatozoa (73-75). Phosphorylation on tyrosine residues occurs during capacitation, interaction of spermatozoa with zona proteins and with progesterone (73-76). The increase in tyrosine phosphorylation during capacitation is time-dependent (Fig. 2).

Such phosphorylation has functional consequence. Incubation of spermatozoa with antiphosphotyrosine antibodies or inhibition of tyrosine kinase activity inhibited zona-free hamster egg penetration (73), prevented acrosome reaction (77) and blocked fertilization (77). However, erbstatin, a potent inhibitor of tyrosine kinase, did not

inhibit capacitation measured as the ability of spermatozoa to respond to progesterone (20). 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 particularly increased the number of sperm cells phosphorylated in the acrosomal region of the sperm head (73). The site of phosphotyrosine-specific fluorescence shifted from the tail of non-capacitated sperm to the acrosome of capacitated spermatozoa (73). There are only a few membrane proteins on the sperm cell that are phosphorylated on tyrosine residues. Among these, the highest degree of tyrosine phosphorylation was found in a protein of 95-97 kDa (73-75). A phosphoprotein in this molecular weight range was found to be the sperm receptor for ZP3, ZRK. This protein has been characterized, partially cloned and sequenced. Sequence shows a 55% homology with the receptor-like protein tyrosine kinase c-eyk (75) and 97-100% homology with the proto-oncogene c-mer (78). This protein has been found only in mature spermatozoa and testicular germ cells (75). A mouse sperm tyrosine phosphorylated protein, migrating at 95 kDa in non reducing conditions, has been sequenced (79). This protein shows complete amino acid homology to a mouse hepatoma hexokinase (HK1) (79). Indeed, the purified sperm protein reacted with an antiserum to the purified rat brain hexokinase type 1 (79). This protein could also be immunoprecipitated with an anti-phosphotyrosine antibody (79). The tyrosine phosphorylated form of HK1 is apparently present only in sperm and testis (80). HK1 is located on the sperm head, has an extracellular domain and behaves like an integral membrane protein (80). Although this protein appears to be phosphorylated, its level of phosphorylation does not change during capacitation in the mouse (19,38). On the other hand, an antibody against ZRK, immunoprecipitated a 95 kDa phosphotyrosine containing protein in human spermatozoa that could not be recognized by an antibody to hexokinase (75). Similarly, a different anti-ZRK antibody, LL95, that bound to the acrosomal region of mouse sperm and which could mimic the effects of ZP3 did not recognize the hexokinase (81). In addition, the anti-hexokinase antibody, although bound to sperm tail region, failed to demonstrate any effect on sperm-zona binding or to stimulate the acrosome reaction (81). Taken together, these data suggest that p95 hexokinase is not involved in sperm-oocyte interaction (81).

During capacitation, the role of several regulating agents in tyrosine kinase activity and protein tyrosine phosphorylation has been studied. In spermatozoa of mouse epididymus, the increase of protein tyrosine phosphorylation during capacitation appeared to be dependent on the presence of BSA, calcium and bicarbonate in the medium (19). The capacitation-inducing activity of these factors was completely dependent on the generation of cAMP (38,82). Addition of cAMP agonists could restore capacitation-induced protein tyrosine phosphorylation in the absence of any of these factors (38). In human spermatozoa, the requirement of calcium ions in the medium for capacitation is different from that observed in the mouse. Depletion of Ca^{2+} in the medium potentiates, rather than inhibits, the protein tyrosine phosphorylation during capacitation of human spermatozoa (20). Presence of calcium chelating agents in the capacitating medium is incapable to decrease such enhanced tyrosine phosphorylation. Moreover, increasing intracellular calcium with calcium ionophores leads to a decrease in the tyrosine phosphorylation of sperm proteins (20).

Two thymosin peptides, Tα1 and Tβ4 were detected in the seminal plasma of men and in the follicular fluids of women (83): the rate of penetration of zona-free hamster ova by human spermatozoa was increased by Tα1 but not Tβ4 (84). A significantly higher concentration of Tα1 was present in the seminal plasma of fertile rather that infertile men. On the contrary, Tβ4 was higher in seminal plasma of infertile as compared to fertile men (84). Tα1 increases the capacitation of human spermatozoa by enhancing tyrosine phosphorylation of several sperm proteins (84). Two of these proteins with molecular weights of 95 and 51 kDa bind to the zona pellucida (84). The stimulatory effect of Tα1 on phosphorylation is exerted only in membrane protein extracts of non-capacitated spermatozoa (85). Tα1 has no effect on protein tyrosine phosphorylation of capacitated spermatozoa (85). Besides the 95-97 kDa protein indicated above, a 51 kDa protein designated fertilization antigen-1 (FA-1), is considered to be a receptor tyrosine kinase and plays an important role in capacitation (86-88). Treatment of human spermatozoa with an anti-FA-1 monoclonal antibody reduced protein phosphorylation including tyrosine phosphorylation of both 95 and 51 kDa proteins (85).

Tyrosine phosphorylation of sperm proteins is greatly enhanced under oxidizing conditions, and reduced under reducing conditions (89). Moreover, biological response to progesterone is significantly enhanced by stimulation of reactive oxygen species (ROS) generation during capacitation and inhibited by scavengers of hydrogen peroxide (90). Presence of other kinases, including c-ras (91), EGF-receptor tyrosine kinase (92), and the cell-cycle specific cyclin, cdc-2 (93), have been demonstrated in mammalian spermatozoa. The role of these enzymes in capacitation is not yet clear. However, the cdc-2 serine/threonine protein kinase, is expressed at a higher level in capacitated spermatozoa, suggesting a possible role of this protein in the process (93).

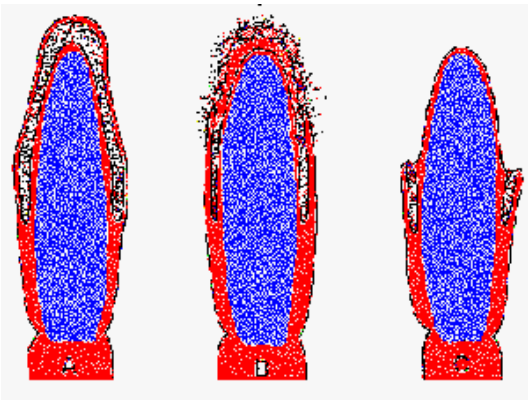

Figure 3. Diagram illustrating the process of acrosome reaction. **(A)** Intact spermatozoon. **(B)** Acrosome reaction in progress: fusion and fenestration of plasma and acrosomal membrane allows the release of acrosomal contents (hydrolyzing enzymes). **(C)** Acrosome-reacted spermatozoon. From (101) with permission.

3. THE ACROSOME REACTION (AR): BACKGROUND

The sperm acrosome, a Golgi-derived structure forming a cap over the anterior region of the nucleus, contains many hydrolytic enzymes (1). Acrosome consists of an anterior cap and a posterior region called equatorial segment (94). During capacitation, the spermatozoa acquires the ability to penetrate the cumulus oophorus and to bind to the zona pellucida. This binding triggers AR which consists of development of multiple fenestrations between the outer acrosomal membrane and the plasma membrane of the spermatozoa (1). This interaction leads 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) (Fig. 3). This exocytotic process, involves the anterior region of sperm head and is not extended beyond the equatorial segment (1). In the absence of any specific stimuli, human spermatozoa can undergo acrosome reaction at a low level (95). It has been suggested that self aggregation of sperm receptor for zona pellucida may account for this spontaneous acrosome reaction (96). According to another hypothesis, the Na^+ and/or Ca^{2+}- pumping mechanisms become less efficient with time (due to depletion of ATP, for example) (1). This would result in a gradual increase in intracellular Ca^{2+} and pH which leads to spontaneous acrosome reaction (1). Molecules such as ATP (97-98) and phosphodiesterase inhibitors like caffeine and pentoxifylline (99-100) and progesterone (101) stimulate the AR *in vitro*. ZP3, a sulphated glycoprotein in the zona pellucida of mammals, is the egg protein that physiologically induces AR (1). Other potential stimuli of AR *in vivo* include the oviductal and follicular fluids as well as several proteins in the cumulus oophorus matrix (Table 1). It is conceivable that the high concentration of progesterone and other steroids in the cumulus oophorus induces AR during the fertilization process. Solubilized zona pellucida and progesterone synergistically induce AR in the mouse and porcine spermatozoa *in vitro* (110,111). Progesterone in the cumulus matrix may "prime" spermatozoa subsequently to their interaction with ZP3 and before they have fully undergone the AR (110). Higher binding of human spermatozoa pre-treated with human follicular fluid to the zona pellucida is consistent with this hypothesis (112).

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

AGONIST	REF
Zona pellucida protein (ZP3)	102
Serum albumin	103
Epidermal growth factor	104
Atrial natriuretic peptide	105, 106
Platelet-activating factor	107, 108
Progesterone & 17αOH-progesterone	101, 109

One of the first events that occurs in spermatozoa following stimulation with ZP3 and progesterone is receptor aggregation (102, 113-114). This is followed by a cascade of downstream membrane and cytosolic signaling factors involved in induction of AR (Fig. 4). Among them, the roles of calcium, phospholipases and protein kinases are discussed below.

3.1. Increase in intracellular calcium during acrosome reaction.

Calcium plays a central role in receptor-mediated response and membrane fusion processes in spermatozoa (1,115). Calcium ionophores are indeed the most widely used non physiological inducers of AR (1). However, AR can be induced in the absence of extracellular calcium with some agonists (97,108,116), whereas extracellular calcium in the millimolar range is essential to the exocytosis induced by ZP3 (25).

Presence of calcium channels was originally shown in the plasma membrane of sea urchin spermatozoa (117). Using the fluorescent probe fura-2, the amount of intracellular calcium in response to addition of ZP3 was studied in mammalian spermatozoa (28, 118). Addition of ZP3 led to a rapid (2-5 min) increase in the amount of intracellular calcium. This was followed by a plateau phase lasting

10-15 min (28, 118). Acrosome reaction occurred during the sustained phase of calcium increase (119). Activation of calcium channels by ZP3 involves pertussin toxin sensitive GTP-binding proteins (118). Ca^{2+} influx stimulated by ZP3 involves specific L-type voltage-dependent plasma membrane Ca^{2+}-channels (28). Ca^{2+}-channel blockers blunt the increase in the intracellular calcium and the AR induced by zona pellucida (28).

Increase of intracellular Ca^{2+} is associated with an efflux of H^+ and a rise in intracellular pH (118). ZP3 also activates non-selective cation channels that lead to membrane depolarization and opening of voltage-sensitive Ca^{2+}-channels (119).

Similar to ZP3, progesterone and follicular fluid induce an influx of calcium in human sperm (120-122). However, in this case, pertussin toxin sensitive GTP-binding proteins and voltage-sensitive calcium channels do not appear to be involved (101,123). Extracellular calcium seems to be required for induction of exocytotic event by progesterone (101). Some data suggest that calcium may be stored in mammalian sperm (23-24,124). For example, thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase induced an influx of calcium (23) and led to AR in the capacitated human sperm (124-125). These effects were dependent on the presence of extracellular calcium (23,125). According to one hypothesis, thapsigargin induces release of calcium from intracellular stores (126). This, in turn, leads to a massive influx of extracellular calcium (126). Spermatozoa do not posses endoplasmic reticulum. Therefore, it has been postulated that such storage sites may exist in the nuclear envelope or in the outer acrosome membrane as suggested by recent data (23, 124-125). For example, the newly identified and characterized IP3 receptors on the outer acrosome membrane of rat sperm may play a role in Ca^{2+} storage (124). Similarly, calreticulin, a calcium binding endoplasmic reticulum protein involved in calcium release, has been recently described in the rat acrosome (127).

Calcium may play a role in the fusion events in the sperm membrane (128). Using a pyroantimonate-osmium fixation technique, the temporal and spatial location of intracellular calcium granules was monitored during acrosome reaction in ram spermatozoa (128). 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 (128)

Fluxes of Na^+ (25,129), Cl^- (130-134), bicarbonate (135) and H^+ (25,119,129) occur during AR, suggesting that, besides Ca^{2+} , other ions may be implicated in the process of fusion of the outer acrosomal membrane and the plasma membrane of spermatozoa.

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 (136). This finding was consistent with activation of phospholipases. In fact, increase of intracellular Ca^{2+} stimulated by ionophores and progesterone leads to activation of phospholipase C in human spermatozoa (120,137-138). Such activation leads to an intracellular increase in the amount of inositol trisphosphate and diacylglycerol. Similarly, ZP3 activates the phosphoinositide specific enzyme phospholipase Cγ (55) by virtue of tyrosine phosphorylation and leads to its translocation from cytosol to particulate fractions (55). Presence and activation of phosphatidylcholine-specific phospholipase C during AR has been also demonstrated in the mammalian spermatozoa (139-140).

Similarly, the AR induced by ionophores and progesterone leads to the activation of phospholipase A2 (141-143). 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 (144-149) 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, in the mammalian spermatozoa (150-151). This phospholipid, that is synthesized during AR (143), may further enhance AR and sperm motility (108,152-155). During AR, phospholipase D is activated and phosphatidic acid is generated (156-158). However, activation of phospholipase D represents a late event in the AR process, and does

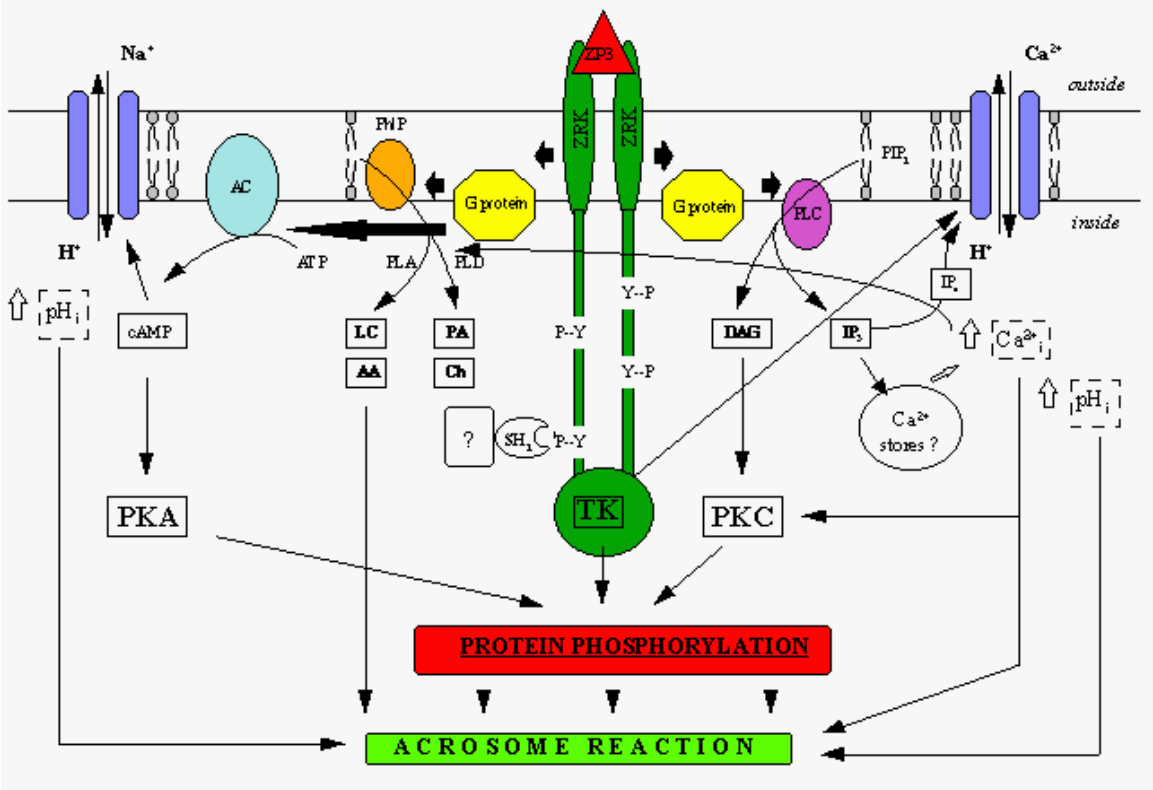

Figure 4. Diagram illustrating the main signaling pathways so far described to be involved in the process of acrosome reaction in response to zona protein 3 (**ZP3**). Following interaction with the agonist, aggregation of receptors for ZP3 (**ZRK**) induces **TK** activation (which increases **protein tyrosine phosphorylation**) and autophosphorylation of the receptor. 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 (**IP_3**) 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. IP_3 may increase intracellular Ca^{2+} by liberation of the ion from intracellular Ca^{2+} stores (not demonstrated in sperm). 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 (**pH_i**). Partial dependence of Ca^{2+}-influx from **TK** activation has been reported. Ca^{2+}-dependent activation of phospholipase A 2 (**PLA_2**) 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 (**Phlp**)] have also been described to occur during acrosome reaction.

not appear to substantially contribute to this event (157-158).

3.3. Activation of protein kinases during acrosome reaction.

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. Preliminary evidence for the involvement of PKA in the AR, demonstrated that adenylate cyclase activity and cAMP generation increases during this process (159). Adenylcyclase agonist, forskolin, and the cAMP analogue, dibutyryl cAMP, induce AR in adose-dependent manner (159-161). These findings suggest that PKA may be involved in AR. PKC activators such as phorbol esters and synthetic DAG, induce acrosome reaction (68,71). Controversial data were reported regarding the strict dependence of this effect on presence of extracellular calcium (68-71). Bielfeld *et al.* (162), showed that stable agonists of cAMP and cGMP, as well as phorbol esters can induce AR in capacitated spermatozoa. This induction did not require presence of extracellular calcium. Similarly, stimulation of kinase activity by zona pellucida does not require extracellular calcium (162). Induction of AR by solubilized zona pellucida

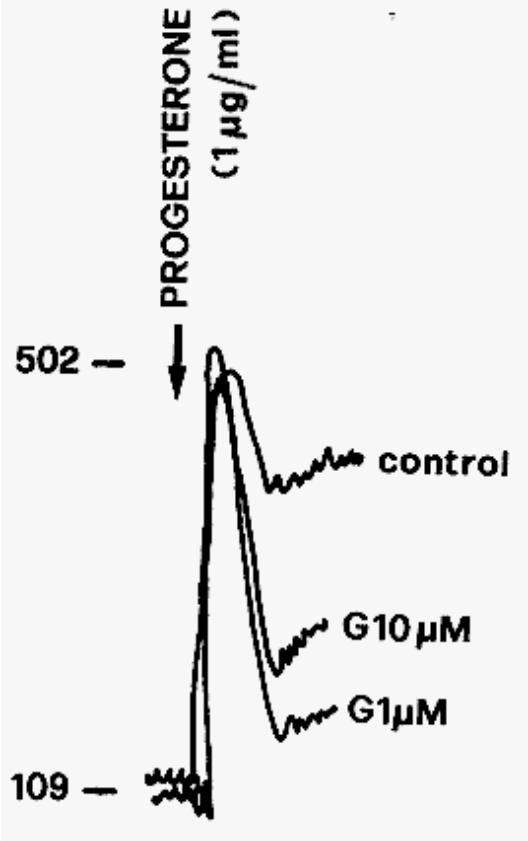

Figure 5: Effect of increasing concentrations of the tyrosine kinase inhibitor genistein on the increase of $[Ca^{2+}]i$ elicited by progesterone in human spermatozoa loaded with fura 2. The peak phase is unaffected, whereas the plateau phase is strongly blunted. From Bonaccorsi et al. (170) with permission.

was partially reduced by pretreatment with inhibitors of PKA, PKC and PKG tested separately, while combination of them caused a significantly greater inhibition. These results suggest an important, concomitant role for PKA, PKC and PKG in human ZP-induced acrosome reaction (162). Combination of PKA and PKC stimulators at their ED_{50} induced an AR equivalent to that obtained with a single stimulator at its ED_{max}, while the combination of the two agents at their ED_{max} was not additive (161). Taken together these results suggest that although the two signalling pathways are partially independent, cross-talks between these pathways are likely to occur. Inhibitors of PKC counteract the effect of progesterone on AR (163).

ZP3 induced an increase in tyrosine phosphorylation of sperm proteins (72,75,77). A major tyrosine phosphorylated protein in mammalian spermatozoa was 95-97 kDa (72-76). This protein appears to undergo autophosphorylation during capacitation and in response to inducers of AR such as ZP3 (72,75) and progesterone (74,76,164). In addition to the 95 kDa receptor protein for ZP3 (75) other sperm binding proteins have been characterized. These include galactosyltransferase (165) and sp56 (166-167) in the mouse, and the FA-1 antigen in the human (168). The inhibitors of tyrosine kinase, genistein and Tyrphostin 47, block ZP3-induced AR (77). Moreover, the tyrosine kinase inhibitor, tyrphostin A48, and pertussis toxin, both inhibit the ZP3-induced calcium influx in the mouse spermatozoa (169). Using a similar pharmacological approach, Tesarik *et al*. (76) and Luconi *et al*. (74) showed involvement of tyrosine kinases in the progesterone-mediated acrosome reaction. Activation of tyrosine kinase seems to be involved in the plateau phase of increase in the amount of intracellular Ca^{2+} in response to progesterone (170-171) (Fig. 5).

Reactive oxygen species may be involved in the regulation of tyrosine phosphorylation in human spermatozoa (89-90). It has been suggested that the increase in tyrosine kinase activity by free radical during capacitation in human spermatozoa improves the response of human spermatozoa to progesterone stimulation (90).

4. Perspective

In vitro capacitation has allowed determining a number of biochemical events that occur in human spermatozoa. It is not clear whether the same or similar events occur during capacitation that takes place *in vivo*. However, progesterone and platelet-activating factor, present in the follicular fluid and/or cumulus matrix, could facilitate or prime the acrosome reaction *in vivo*. The responsiveness of spermatozoa to progesterone might be used as a routine clinical test (172-175). Based on such a test, males can be separated into those with normal or reduced sperm response to progesterone (175). Based on such data, the decision can be made to either perform *in vitro* fertilization or to resort to a more invasive technique such as intracytoplasmatic sperm injection (ICSI).

5. ACKNOWLEDGMENTS

We thank Prof. Mario Serio (Endocrinology Unit, University of Florence) for revision of the manuscript. Supported by Consiglio Nazionale delle Ricerche (CNR, Rome - Progetto FATMA), Ministero dell'Universita' e della Ricerca Scientifica and Regione Toscana.

6. REFERENCES

1. R. Yanagimachi. Mammalian fertilization. In: *Physiology of Reproduction*. Eds: Knobil E., Neill J. Raven Press, New York 189-317 (1994)

2. H. M. Florman: Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. *Dev Biol* 165, 152-164 (1994)

3. E. Baldi, R. Casano, C. Falsetti, Cs. Krausz, M. Maggi & G. Forti: Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl* 12, 323-330 (1991)

4. C. Mendoza & J. Tesarik: A plasma-membrane progesterone receptor in human sperm is switched on by increasing intracellular free calcium. *FEBS Lett* 330, 57-60 (1993)

5. S. A. Meyers, J. W. Overstreet, I. K. M. Liu & E. Z. Drobnis: Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reaction in fertile and subfertile males. *J Androl* 16, 47-54 (1995)

6. A. Cohen-Dayag & M. Eisenbach: Potential assay for sperm capacitation in mammalians. *Am J Physiol* 267, c1167-c1176 (1994)

7. J. Stewart-Savage: Effect of bovine serum albumin concentration and source on sperm capacitation in the golden hamster. *Biol Reprod* 49, 74-81 (1993)

8. A. Cohen-Dayag, I. Tur-Kaspa, J. Dor, S. Mashiach & M. Eisenbach: Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. *Proc Natl Acad Sci USA* 92, 11039-11043 (1995)

9. R. Zhou, B. Sky, K. C. K. Chou, M. D. Oswald & A. Hang: Changes in intracellular calcium of porcine sperm during *in vitro* incubation with seminal plasma and a capacitation medium. *Biochem Biophy Res Commun* 172, 47-53 (1990)

10. D.R. White & R. J. Aitken: Relationship between calcium, cyclic AMP, ATP and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. *Gamete Res* 22, 163-177 (1989)

11. C. E. Coronel & H. A. Lardy: Characterization of Ca^{2+} uptake by guinea pig epididymal spermatozoa. *Biol Reprod* 37, 1097-1107 (1987)

12. L. R. Fraser & C. A. McDermott: Ca^{2+}-related changes in the mouse sperm capacitation state: a possible role for Ca^{2+}-ATPase. *J Reprod Fertil* 96, 363-377 (1992)

13. N. Okamura, M. Tanba, A. Fukuda, Y. Sugita & T. Nagai: Forskolin stimulates porcine sperm capacitation by increasing calcium uptake. *FEBS Lett* 316, 283-286 (1993)

14. S. S. Suarez, S. M. Varosi & X. Dai: Intracellular calcium increases with hyperactivation in intact moving hamster sperm and oscillates with the flagellar beat cycle. *Proc Natl Acad Sci USA* 90, 4660-4664 (1993)

15. S. DasGupta, C. L. Millis, & L. R. Fraser: Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. *J Reprod Fertil* 99, 135-143 (1993)

16. L. R. Fraser: Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization *in vitro*. *J Reprod Fertil* 81, 77-89 (1987)

17. E. R. S. Roldan & A.D. Fleming: Is a Ca^{2+}-ATPase involved in Ca^{2+} regulation during capacitation and the acrosome reaction of guinea-pig spermatozoa? *J Reprod Fertil* 85, 297-308 (1989)

18. C. E. Stock & L. R. Fraser: Divalent cations, capacitation and the acrosome reaction in human spermatozoa. *J Reprod Fertil* 87, 463-478 (1989)

19. P. E. Visconti, J. L. Ballay, G. D. Moore, D. Pan, P. Olds Clarke & G. S. Kopf: Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 121, 1139-1150 (1995)

20. M. Luconi, E. Baldi, Cs. Krausz & G. Forti: Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod* 55, 207-216 (1996)

21. L. R. Fraser & C. A. McDermott: Ca^{2+}-related changes in the mouse sperm capacitation state: a possible role for Ca^{2+}-ATPase. *J Reprod Fertil* 96, 363-377 (1992)

22. L. R. Fraser: Mechanisms regulating capacitation and the acrosome reaction. In: *Human sperm acrosome reaction.* Eds: Fenichel P., Parinaud J., Colloque/INSERIM John Libbey Eurotext Ltd, Montrouge, France, vol. 236, 17-33 (1995)

23. P. F. Blackmore: Thapsigargin elevates and potentiates the ability of progesterone to increase intracellular free calcium in human sperm: possible role of perinuclear calcium. *Cell Calcium* 14, 53-60 (1992)

24. C. Serres, J. Yang & P. Jouannet: RU486 and calcium fluxes in human spermatozoa. *Biochem Biophys Res Commun* 204, 1009-1015 (1994)

25. S. A. Adeoya-Osiguwa & L. R. Fraser: A byphasic pattern of $^{45}Ca^{2+}$ uptake by mouse spermatozoa *in vitro* correlates with changing functional potential. *J Reprod Fertil* 99, 187-194 (1993)

26. L. R. Fraser, L. R. Abeydeera & K. Niwa: Ca^{2+}-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Mol Reprod Dev* 40, 233-241 (1995)

27. R. J. Mrnsy, J. E. Siiteri & S. Meizel: Hamster sperm Na^+, K^+-adenosine triphosphatase: increased activity during capacitation of murine epidydimal sperm. *Biol Reprod* 30, 573-584 (1984)

28. H. M. Florman, M. E. Corron, T. D.-H. Kim, D. Babcock: Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev Biol* 152, 304-314 (1992)

29. C. Beltran, A. Darscon, P. Labarca & A. Lievano: A high-conductive voltage-dependent multistate Ca^{2+} channels found in sea urchin and mouse spermatozoa. *FEBS Lett* 338, 23-26 (1994)

30. Y. Zeng, E. N. Clark & H. M. Florman: Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Dev Biol* 71, 554-563 (1995)

31. R. V. Hyne, K. P. Edwards & J. D. Smith: Changes in guinea pig sperm intracellular sodium and potassium content during capacitation and treatment with monovalent ionophore. *Gamete Res* 12, 65-73 (1985)

32. W. L. Vredenburgh-Wilberg & J. J. Parrish: Intracellular pH of bovine sperm increases during capacitation. *Mol Reprod Dev* 40, 490-592 (1995)

33. J. M. Neill & P. Olds-Clarke: A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. *Gamete Res* 18, 121-140 (1987)

34. D. E. Boatmen. & R. S. Robbins: Bicarbonate:carbone-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod* 44, 806-813 (1991)

35. M. A. Lee. & B. T. Storey: Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. *Biol Reprod* 34, 349-356 (1986)

36. Q.-X. Shi & E. R. S. Roldan: Bicarbonate/CO2 is not required for zona pellucida- or progesterone-induced acrosomal exocytosis of mouse spermatozoa but is essential for capacitation. *Biol Reprod* 52, 540-546 (1995)

37. L. R. Fraser. & N. J. Monks: Cyclic nucleotides and mammalian sperm capacitation. *J Reprod Fertil* 42, 9-21 (1990)

38. P. E. Visconti, G. D. Moore, J. L. Balley, P. Leclerc, S. A. Connors, D. Pan, P. Olds-Clarke & G. S. Kopf: Capacitation in mouse spermatozoa. II Tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 121, 139-1150 (1995)

39. J. C. Andrews, J. P. Nolan, R. H. Hammerstedt & B. D. Bavister: Role of zinc during hamster sperm capacitation. *Biol Reprod* 51, 1238-1247 (1994)

40. K. J. Go & D. P. Wolf : The role of sterols in sperm capacitation. *Adv Lip Res* 20, 317-330 (1983)

41. K. J. Go & D. P. Wolf: Albumin-mediated changes in the sperm sterol contents during capacitation. *Biol Reprod* 32, 145-153 (1985)

42. J. Langlais & K. D. Roberts: A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res* 12, 183-224 (1985)

43. E. Ehrenwald, R. H. Foote & J. E. Parks: Bovine oviductal fluid components and their potential role in sperm cholesterol efflux. *Mol Reprod Dev* 25, 195-204 (1990)

44. E. Ehrenwald, J. E. Parks & R. H. Foote: Cholesterol efflux from bovine sperm. I. *Gamete Res* 20, 145-157 (1988)

45. K. Hoshi, T. Aita, K. Yanagida, N. Yoshimatsu & A. Sato: Variation in the cholesterol/phospholipid ratio in human spermatozoa and its relation with capacitation. *Hum Reprod* 5, 1-74 (1990)

46. S. Benoff: The role of cholesterol during capacitation of human spermatozoa. *Hum Reprod* 8, 2001-2008 (1993)

47. B. K. Davis, R. Byrne & B. Hungund: Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation *in vitro*. *Biochem Biophys Acta* 558, 257-266 (1979)

48. B. K. Davis, R. Byrne & K. Bedigan: Albumin-mediated changes in plasma membrane lipids during *in vitro* capacitation. *Proc Natl Acad Sci USA* 77, 1546-1550 (1980)

49. N. Tanphaichitr, Y. S. Zheng, M. Kates, N. Abdullah & A. Chan: Cholesterol and phospholipid levels of washed and percoll gradient centrifuged mouse sperm: presence of lipids possessing inhibitory effects on sperm motility. *Mol Reprod Dev* 43, 187-195 (1996)

50. C. H. Muller & S. E. Ravnik: Lipid transfer protein: a natural stimulator of the sperm capacitation process. In: *Human Sperm Acrosome Reaction.* Eds: Fenichel P., Parinaud J., Colloque/INSERIM John Libbey Eurotext Ltd, Montrouge, France, vol. 236, 67-84 (1995)

51. M. N. Llanos & S. Meizel: Phospholipid methylation increases during capacitation of golden hamster sperm *in vitro*. *Biol Reprod* 28, 1043-1051 (1983)

52. R. A. P. Harrison & B. M. Gadella: Membrane changes during capacitation with special reference to lipid architecture. In: *Human sperm acrosome reaction.* Eds: Fenichel P., Parinaud J., Colloque INSERIM/John Libbey Eurotext Ltd, Montrouge, France, vol. 236, 45-65 (1995)

53. D. R. Snider & E. D. Clegg: Alteration of phospholipids in porcine spermatozoa during *in vivo* uterus and oviduct incubation. *J Anim Sci* 40, 269-274 (1975)

54. E. A. Dennis: Diversity of group types, regulation and function of phospholipase A2. *J Biol Chem* 269, 13057-13060 (1994)

55. M. R. Fry, S. S. Ghosh, J. M. East & R. C. Franson: Role of human sperm phospholipase A2 in fertilization: effects of a novel inhibitor of phospholipase A2 activity on membrane perturbations and oocyte penetration. *Biol Reprod* 47, 751-759 (1992)

56. C. N. Tomes, C. R. McMaster & P. M. Saling: Activation of mouse PIP2-PLC by zona pellucida is modulated by tyrosine phosphorylation. *Mol Reprod Dev* 43, 196-204 (1996)

57. B. Margolis, S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein & J. Schlessinger: EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* 57, 1101-1107 (1989)

58. J. Meisenhelder, P.-G. Suh, S. G. Rhee & T. Hunter: Phospholipase C is a substrate for PDGF and EGF receptor protein tyrosine kinases *in vivo* and *in vitro*. *Cell* 57, 1109-1122 (1989)

59. S. K. Hanks & T. Hunter: The eukariyotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* 9, 576-596 (1995)

60. D. M. Stein & L. R. Fraser: Cyclic nucleotide metabolism in mouse epidydimal spermatozoa during capacitation *in vitro*. *Gamete Res* 10, 283-299 (1984)

61. N. J. Monks, D. M. Stein & L. R. Fraiser: Adenylate cyclase activity of mouse sperm during capacitation *in vitro*: effect of calcium and a GTP analogue. Int *J Androl* 9, 67-76 (1986)

62. T. Berger & E. D. Clegg: Adenylate cyclase activity in porcine sperm in response to female reproductive tract secretions. *Gamete Res* 7, 169-177 (1983)

63. J. J. Parrish, J. L. Susko-Parrish, C. Uguz & N. L. First: Differences on the role of cyclic adenosine 3,5-monophosphate during capacitation of bovine sperm by heparin or oviductal fluid. *Biol Reprod* 51, 1099-1108 (1994)

64. V. J. Kay, J. R. T. Coutts & L. Robertson: Effect of pentoxifylline and progesterone on human sperm capacitation and acrosom reaction. *Hum Reprod* 9, 2318-2323 (1994)

65. D. L. Gerbers, D. J. Tubb & R. V. Hyne: Requirement of bicarbonate for Ca^{2+}-induced elevations of cyclic AMP in guinea pig spermatozoa. *J Biol Chem* 257, 8980-8984 (1982)

66. R. Rotem, G. F. Par, Z. T. Homonnai, M. Kalina & Z. Naor: Protein kinase C is present in human sperm: possible role on flagellar motility. *Proc Natl Acad Sci USA* 87, 7305-7308 (1990)

67. H. Breitbart, J. Lax, R. Rotem & Z. Naor: Role of protein kinase C in the acrosome reaction of mammalian spermatozoa. *Biochem J* 281, 473-476 (1992)

68. Rotem, G. F. Paz, Z. T. Homonnai, M. Kalina, J. Lax, H. Breitbart & Z. Naor: Ca^{2+}-independent induction of acrosome reaction by protein kinase C in human sperm. Endocrinology 131, 2235-2243 (1992)

69. S. Furuya, Y. Endo, M. Oba & S. Suzuki: Effects of modulators of protein kinase C on human sperm capacitation. *Fertil Steril* 59, 1285-1290 (1993)

70. S. Furuya, Y. Endo, M. Oba, S. Suzuki & S. Nozawa: Effect of epidermal growth factor on human sperm capacitation. *Fertil Steril* 60, 905-910 (1993)

71. C. De Jonge, H. L. Han, S. R. Mack, J. D. Zaneveld: Effect of phorboldiesters synthetic diacylglycerols and a protein kinase C inhibitor on

the human sperm acrosome reaction. *J Androl* 12, 62-70 (1991)

72. L. Leyton & P. Saling: 95 kd sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. *Cell* 57, 1123-1130 (1989)

73. R. K. Naz, K. Ahmad & R. Kumar: Role of membrane phosphotyrosine proteins in human spermatozoa function. *J Cell Sci* 99, 157-165 (1991)

74. M. Luconi, L. Bonaccorsi, Cs. Krausz, G. Gervasi, G. Forti & E. Baldi: Stimulation of protein tyrosine phosphorylation by platelet-activating factor and progesterone in human spermatozoa. *Mol Cell Endocrinol* 108, 35-42 (1995)

75. D. J. Burks, R. Carballada, H. D. M. Moore & P. M. Saling: Interaction of a tyrosine kinase from human sperm with the zona pellucida at fertilization. *Science* 269, 83-86 (1995)

76. J. Tesarik, J. Moos & C. Mendoza: Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm. *Endocrinol* 133, 328-335 (1993)

77. L. Leyton, P. LeGuen, D. Bunch, P. M. Saling: Regulation of mouse gamete interaction by sperm tyrosine kinase. *Proc Natl Acad Sci USA* 89, 11692-11695 (1992)

78. P. Bork, J.-Y. Tsai / L. M. Silver / P. Saling, R. Carballada, D. Burks & H. Moore: Sperm-egg binding protein or proto-oncogene ?. *Science* 271, 1431-1435 (1996)

79. P. Kalab, P. E. Visconti, P. Leclerc & G. S. Kopf: p95, the major phosphotyrosine-containing protein in mouse spermatozoa, is a hexokinase with unique properties. *J Biol Chem* 269, 3810-3817 (1994)

80. P. Visconti, P. Olds-Clarke, S. B. Moss, P. Kalab, A. J. Travis, M. De Las Heras & G. S. Kopf: Properties and localization of a tyrosine phosphorylated form of hexokinase in mouse sperm. *Mol Reprod Dev* 43, 82-93 (1996)

81. L. Leyton, C. Tomes & P. Saling: LL95 monoclonal antibody mimics functional effects of ZP3 on mouse sperm: evidence that the antigen recognized is not hexokinase. *Mol Reprod Dev* 42, 347-358 (1995)

82. A. E. Duncan & L. R. Fraser: Cyclic AMP-dependent phosphorylation of epididymal mouse sperm proteins during capacitation *in vitro*: identification of an Mr 95000 phosphotyrosine-containing protein. *J Reprod Fertil* 97, 287-299 (1993)

83. R. K. Naz, P. H. Naylor & A. L. Goldstein: Thymosin 1 levels in human seminal plasma and follicular fluid: implication in germ cell function. *Int J Fertil* 32, 375-379 (1989)

84. R. K. Naz, P. Kaplan & A. L. Goldstein: Thymosin alpha-1 enhances the fertilizing capacity of human sperm cell: implication in diagnosis and treatment of male infertility. *Biol Reprod* 47, 1064-1072 (1992)

85. R. K. Naz: Role of membrane tyrosine kinases in human sperm function. In: *Human Sperm Acrosome Reaction.* Eds: Fenichel P., Parinaud J., Colloque/INSERIM John Libbey Eurotext Ltd, Montrouge, France, vol. 236, 245-255 (1995)

86. R. K. Naz, N. J. Alexander, M. Isahakia & M. S. Hamilton: Monoclonal antibody to a human sperm membrane glycoprotein that inhibits fertilization. *Science* 225, 342-344 (1984)

87. P. Kaplan. & R. K. Naz: The fertilization antigen-1 does not have any proteolytic/acrosin activity, but its monoclonal antibody inhibits sperm capacitation and acrosome reaction. *Fertil Steril* 58, 396-402 (1992)

88. R. K. Naz, C. Morte, V. Garcia-Framis, P. Kaplan & P. Martinez: Characterization of a sperm-specific monoclonal antibody and isolation of a 95-kilodalton fertilization antigen-2 from human sperm. *Biol Reprod* 49, 1236-1244 (1993)

89. R. J. Aitken, M. Paterson, H. Fisher, D. W. Buckingham & M. van Duin: Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* 108, 2017-2025 (1995)

90. R. J. Aitken, D. W. Buckingham, D. Harkiss, M. Paterson, H. Fisher & D. S. Irvine: The extragenomic action of progesterone on human spermatozoa is influenced by redox regulated changes in tyrosine phosphorylation during capacitation. *Mol Cell Endocrinol* 117, 83-93 (1996)

91. R. K. Naz, K. Ahmad & P. Kaplan: Expression and function of ras-proto-oncogene proteins in human sperm cells. *J Cell Sci* 102, 487-494 (1992)

92. R. K. Naz & K. Ahmad: Presence of expression products of c-erbB-1 and c-erbB-2/HER2 genes on mammalian sperm cell, and effects of their regulation on fertilization. *J Reprod Immunol* 21, 223-239 (1992)

93. R. K. Naz, K. Ahmad & P. Kaplan: Involvement of cyclins and cdc2 serine/threonine protein kinase in human sperm cell function. *Biol Reprod* 48, 720-728 (1993)

94. E. M. Eddy & D.A. O'Brien: The spermatozoon. In: *The Physiology of Reproduction*. Eds: Knobil E., Neill J. D. New York: Raven; pages 29-77 (1994)

95. C. E. Stock & L. R. Fraser: The acrosome reaction in human sperm from men of proven fertility. *Hum Reprod* 2, 109-114 (1987)

96. P. M. Saling: Mammalian sperm interaction with extracellular matrices of the egg. In: *Oxford Review of Reproductive Biology*, Ed: Milligan S.R., Oxford: Oxford University Press; pages:339-388 (1989)

97. C. Foresta, M. Rossato & F. Di Virgilio: Extracellular ATP is a trigger for the acrosome reaction in human spermatozoa. *J Biol Chem* 267, 19443-19447 (1993)

98. T. Tomiyama, K. Ohashi, T. Tsutsui, F. Saji, & O. Tanizawa: Acrosome reaction induced in a limited population of human spermatozoa by progesterone (Ca^{2+}-dependent) and ATP (Ca^{2+}-independent) *Hum Reprod* 10, 2052-2055 (1995)

99. J. Tesarik & C. Mendoza: Sperm treatment with pentoxifylline improves fertilizing ability in patients with acrosome reaction insufficiency. *Fert Steril* 60, 141-48 (1995)

100. S. DasGupta, C. O'Toole, C. L. Mills & L. R. Fraser: Effect of pentoxifylline and progesterone on human sperm capacitation and acrosome reaction. *Hum Reprod* 9, 2103-9 (1994)

101. E. Baldi, Cs. Krausz, & G. Forti: Nongenomic actions of progesterone on human spermatozoa. Trends *Endocrinol* Metab 6, 198-205 (1995)

102. L. Leyton & P. M. Saling: Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J *Cell* Biol 108, 2163-2168 (1989)

103. S. Meizel: Molecules that initiate or help stimulate the acrosome reaction by their interaction with mammalian sperm surface. *Am J Anat* 174, 285-302 (1985)

104. Y. Lax, S. Rubinstein & H. Breitbart: Epidermal growth factor induces acrosomal exocytoosis in bovine sperm. *FEBS Lett* 339, 234-238 (1994)

105. R. A. Anderson, K. A. Feathergill, R. C. Drisdel, R. G. Rawlins, S. R. Mack & L. J. D. Zaneveld: Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with *in vitro* fertilization outcome. *J Androl* 15, 61-70 (1994)

106. N. Zamir, D. Barkan, N. Keynan, Z. Naor & H. Breitbart: Atrial natriuretic peptide induces acrosomal exocytosis in bovine spermatozoa. *Am J Physiol* 269, E216-E221 (1995)

107. M. J. Angle, R. Tom, K. Jarvi & R.D. McClure: Effect of platelet-activating factor (PAF) on human spermatozoa-oocyte interactions. *J Reprod Fertil* 98, 541-548 (1993)

108. Cs. Krausz, G. Gervasi, G. Forti & E. Baldi: Effect of platelet-activating factor on motility and acrosome reaction of human spermatozoa. *Hum Reprod* 9, 471-476 (1994)

109. R. A. Osman, M. L. Andria, A. D. Jones & S. Meizel: Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun* 160, 828-833 (1989)

110. R. S. Roldan, T. Murase & Q-X Shi: Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science* 266, 1578-1581 (1994)

111. C. S. Melendrez, S. Maizel & T. Berger: Comparison of ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction *in vitro*. *Mol Reprod Dev* 39, 433-438 (1994)

112. P. Morales, N. L. Cross, J. W. Overstreet & F. W. Hansson: Acrosome intact and acrosome reacted human sperm can initiate binding to the zona pellucida. *Dev Biol* 133, 385-392 (1989)

113. J. Tesarik, C. Mendoza, J. Moos, P. Fenichel & M. Fehlmann: Progesterone action through aggregation of a receptor on the sperm plasma membrane. *FEBS Lett* 308, 116-20 (1992)

114. J. Tesarik & C. Mendoza: Insights into the function of sperm surface progesterone receptor: Evidence of ligand induced receptor aggregation and implication of proteolysis. *Exp Cell Res* 205, 111-117 (1993)

115. P. Thomas & S. Meizel: An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Res* 20, 397-411 (1988)

116. P. R. Bielfeld, A. Anderson, S. R. Mack, C. J. De Jonge & L. J. D. Zaneveld: Are capacitation or calcium ion influx required for the human sperm acrosome reaction? *Fert Ster* 62, 1255-1261 (1994)

117. A. M. Darzon, A. Guerrero, A. Lievano, M. Gonzales-Martinez & E. Morales: Ionic channels in

sea urchin sperm physiology. *News Physiol Sci* 3, 181-185 (1988)

118. H. M. Florman, R. M. Tombes, N. L. First & D. F. Babcock: An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevations of internal Ca and pH that mediate mammalian sperm acrosomal exocytosis. *Dev Biol* 135, 133-146 (1989)

119. H. M. Florman, J. R. Lemos, C. Arnoult, J. A. Oberdof & Y. Zeng: Exocytotic ion channels in mammalian sperm. In: *Human sperm acrosome reaction.* Eds: Fenichel P., Parinaud J., Colloque/INSERIM John Libbey Eurotext Ltd, Montrouge, France, vol. 236, 179-189 (1995)

120. P. Thomas & S. Meizel: Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem J* 264, 539-546 (1989)

121. P. F. Blackmore, S. J. Beebe, D. R. Danforth & N. Alexander: Progesterone and 17α-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem* 265, 1376-1380 (1990)

122. P. F. Balckmore, J. Neulen, F. Lattanzio & S. J. Beebe: *Cell* surface binding sites for progesterone mediate calcium uptake in human sperm. *J Biol Chem* 266, 18655-18659 (1991)

123. P. F. Blackmore: Rapid non-genomic actions of progesterone stimulate Ca^{2+} influx and acrosome reaction in human sperm. *Cell* Signal 5, 531-538 (1993)

124. L.D. Walensky. & S. H. Snyder: Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J Cell Biol* 130, 857-869 (1995)

125. S. Meizel & K. O. Turner: Initiation of the human sperm acrosome reaction by thapsigargin. *J Exp Zool* 267, 350-355 (1993)

126. J.W. Putney: Capacitative calcium entry revisited. *Cell Calcium* 11, 611-624 (1990)

127. M. Nakamura, M. Moryva, T. Baba, Y. Michikawa, T. Yamanobe, K. Arai, S. Okinaga & T. Kobayashi: An endoplasmic reticulum protein, calreticulin, is transported into the acrosome of rat sperm. *Exp Cell Res* 205, 101-110 (1993)

128. P. F. Watson, J. M. Plummer, P. S. Jones & J. C. S. Bredl: Localization of intracellular calcium during the acrosome reaction in ram spermatozoa. *Mol Reprod Dev* 41, 513-520 (1995)

129. C. Foresta, M. Rossato & F. Di Virgilio: Ion fluxes through the progesterone-activated channel of the sperm plasma membrane. *Biochem J* 294, 279-283 (1993)

130. P. F. Blackmore, W. Bin Im & J. E. Bleasdale: The cell surface progesterone receptor which stimulates calcium influx in human sperm is unlike the A ring reduced steroid site on the $GABA_A$ receptor/chloride channel. *Mol Cell Endocrinol* 104, 237-243 (1994)

131. K. O. Turner, M. A. Garcia & S. Meizel: Progesterone initiation of human acrosome reaction: obligatory increase in intracellular calcium is independent of the chloride requirement. *Mol Cell Endocrinol* 101, 221-225 (1994)

132. A. Aanesen, G. Fried, E. Andersson & C. Gottlieb: Evidence for γ-aminobutyric acid specific binding sites on human spermatozoa. *Hum Reprod* 10, 1885-1890 (1995)

133. C. A. Wistrom. & S. Meizel: Evidence suggesting involvement of a unique human sperm steroid receptor/Cl^- channel complex in the progesterone-initiated acrosome reaction. *Dev Biol* 159, 679-690 (1993)

134. C. S. Melandrez & S. Meizel: Different Chloride channels are involved in the progesterone- and zona-initiated porcine sperm acrosome reaction. *Biol Reprod* 53, 676-683 (1995)

135. K. Sauber & S. Meizel: Importance of bicarbonate to the progesterone-initiated human sperm acrosome reaction. *J Androl* 16, 266-271 (1995)

136. M. Nikolopoulou, D. A. Soucek & J. C. Vary: Modulation of the lipid composition of boar sperm plasma membranes during an acrosome reaction *in vitro*. *Arch Biochem Biophys* 250, 30-37 (1986)

137. P. J. Bennet, J-P. Moatti, A. Mansat, H. Ribbes, J-C. Cayrac, F. Pontonnier, H. Chap & L. Douste-Blazy: Evidence for the activation of phospholipases during acrosome reaction of human sperm elicited by calcium ionophore A23187. *Biochim Biophys Acta* 919, 255-265 (1987)

138. E. R. S. Roldan & R. A. P. Harrison: Polyphosphoinositide breakdown and subsequent exocytosis in the Ca^{2+}/ionophore-induced acrosome reaction of mammalian spermatozoa. *Biochem J* 259, 379-406 (1989)

139. R. G. Sheikhnejad & P. N. Srivastava: Isolation and properties of a phosphatidylcholine-specific

phospholipase C from bull seminal plasma. *J Biol Chem* 261, 7544-7549 (1986)

140. E. R. S. Roldan & T. Murase: Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. *J Biol Chem* 269, 23583-23589 (1994)

141. E. R. S. Roldan & C. Fragio: Phospholipase A2 activation and subsequent exocytosis in the Ca^{2+}/ionophore-induced acrosome reaction of ram spermatozoa. *J Biol Chem* 268, 13962-13970 (1993)

142. E. R. S. Roldan & C. Fragio: Diacylglycerols stimulate phospholipase A2 and subsequent exocytosis in ram spermatozoa. *Biochem J* 297, 225-232 (1994)

143. E. Baldi, C. Falsetti, Cs. Krausz, G. Gervasi, V. Carloni, R. Casano & G. Forti: Stimulation of platelet-activating factor synthesis by progesterone and A23187 in human spermatozoa. *Biochem J* 292, 209-216 (1993)

144. N. L. Cross: Phosphatidylcholine enhances the acrosomal responsiveness of human spermatozoa. *J Androl* 15, 484-488 (1994)

145. A. D. Fleming. & R. Yanagimachi: Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. *Gamete Res* 4, 253-273 (1981)

146. K. Kyono, K. Hoshi, A. Saito, A. Tsuiki, H. Hoshiai & M. Suzuki: Effects of phospholipase A2, lysophosphatidylcholine, and fatty acid on the acrosome reaction of human spermatozoa. *J Exp Med* 144, 257-263 (1984)

147. M-H Lachapelle, R. Bouzayen, J. Langlais, K. Jarvi, J. Bourque & P. Miron: Effect of lysoplatelet-activating factor on human sperm fertilizing ability. *Fertil Steril* 59, 863-868 (1993)

148. S. Meizel & K.O. Turner: Stimulation of an exocytotic event, the hamster sperm acrosome reaction, by cis-unsaturated fatty acids. *FEBS Lett* 161, 315-318 (1983)

149. N. Lapage & K. D. Roberts: Purification of lysophospholipase of human spermatozoa and its implication in the acrosome reaction. *Biol Reprod* 52, 616-624 (1995)

150. R. Kumar, M. J. K. Harper & D. J. Hanahan: Occurrence of platelet-activating factor in rabbit spermatozoa. *Arch Biochem Biophys* 260, 497-502 (1988)

151. B. S. Minhas, R. Kumar, D. D. Richer, J. L. Robertson & M. G. Dodson: The presence of platelet-activating factor-like activity in human spermatozoa. *Fertil Steril* 55, 372-376 (1991)

152. M. J. Angle, R. Tom, K. Jarvi & R. D. McClure: Effect of platelet-activating factor (PAF) on human spermatozoa-oocyte interactions. *J Reprod Fertil* 98, 541-548 (1993)

153. A. Fukuda, W. E. Roudebusk & S. S. Thatcher: Platelet-activating factor enhances the acrosome reaction, fertilization *in vitro* by subzonal sperm injection and resulting embryonic development in the rabbit. *Hum Reprod* 9, 94-99 (1994)

154. K. Sengoku, K. Tamate, Y. Takaoka & M. Ishikawa: Effects of platelet-activating factor on human sperm function *in vitro*. *Hum Reprod* 9, 1443-1447 (1993)

155. W. J. G. Hellstrom, R. Wang & S. C. Sikka: Platelet-activating factor stimulates motion parameters of cryopreserved human spermatozoa. *Fertil Steril* 51, 768-770 (1991)

156. S. E. Domino, S. B. Bokkino & D. L. Garbers: Activation of phospholipase D by the fucose-sulfate glycoconjugate that induces an acrosome reaction in spermatozoa. *J Biol Chem* 264, 9412-9419 (1989)

157. E. R. S. Roldan & R. A. P. Harrison: Diacylglycerol and phosphatidate production and the exocytosis of the sperm acrosome. *Biochem Biophys Res Commun* 172, 8-15 (1990)

158. E. R. S. Rodan & E. N. Dawes: Phospholipase D and exocytosis of the ram sperm acrosome. *Biochim Biophys Acta* 1210, 48-54 (1993)

159. C. De Jonge, H-L. Han, H. Lawrie, S. R. Mack & L. J. D. Zaneveld: Modulation of the human sperm acrosome reaction by effector of adenylate cyclase/cyclic AMP second messanger pathway. *J Exp Zool* 258, 113-125 (1991)

160. R. A. Anderson, K. A. Feathergill, C. J. De Jonge, S. R. Mack & L. J. D Zaneveld.: Facilitative effect of pulsed addition of dibutyryl cAMP on the acrosome reaction of noncapacitated spermatozoa. *J Androl* 13, 398-408 (1992)

161. C. M. Doherty, S. M. Tarchala, E. Radwanska & C. J. De Jonge: Characterization of two second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. *J Androl* 16,36-46 (1995)

162. P. Bielfeld, A. Faridi, L. J. D. Zaneveld & C. J. De Jonge: The zona pellucida-induced acrosome

reaction of human spermatozoa is mediated by protein kinases. *Fertil Steril* 61, 536-541 (1994)

163. C. Foresta, M. Rossato & F. Di Virgilio: Differential modulation by protein kinase C of progesterone-activated responses in human sperm. *Biochem Biophys Res Commun* 206, 408-413 (1995)

164. E. Baldi, Cs. Krausz, M. Luconi, L. Bonaccorsi, M. Maggi & G. Forti: Actions of progesterone on human sperm: a model of non genomic effects of steroids. *J Steroid Biochem Mol Biol* 53, 199-203 (1995)

165. D. J. Miller, M. B. Macek & B. D. Shur: Complementary between sperm surface β-1,4-galactosyl-transferase and egg-coat ZP3 mediates sperm-egg binding. *Nature* 357, 589-593 (1992)

166. A. Cheng, T. Le, M. Palacios, L. H. Bookbinder, P. M. Wassarman, F. Suzuki & J. D. Bleil: Sperm-egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. *J Cell Biol* 125, 867-878 (1994)

167. L. H. Bookbinder, A. Cheng & J. D. Bleil: Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein. *Science*, 269, 86-89 (1995)

168. R. K. Naz & K. Ahmad: Molecular identities of human sperm proteins that bind human zona pellucida: nature of sperm-zona interaction, tyrosine kinase activity and involvement of FA-1. *Mol Reprod Dev* 39, 397-408 (1994)

169. J. L. Bailey & T. S. Bayard: Calcium influx into mouse spermatozoa activated by solubilized mouse zona pellucida, monitored with calcium fluorescent indicator, Fluo-3. Inhibition of the influx by three inhibitors of the zona pellucida induced acrosome reaction: tyrphostin A48, pertussis toxin and 3-quinuclidinyl enzilate. *Mol Reprod Dev* 39, 297-308 (1994)

170. L. Bonaccorsi, M. Luconi, G. Forti & E. Baldi: Tyrosine kinase inhibition reduces the plateau phase of the calcium increase in response to progesterone in human spermatozoa. *FEBS Lett* 364, 83-86 (1995)

171. J. Tesarik, A. Carreras & C. Mendoza: Single cell analysis of tyrosine kinase dependent and independent Ca^{2+} fluxes in progesterone induced acrosome reaction. *Mol Hum Reprod* 2, 225-232 (1996)

172. J. Tesarik & C. Mendoza: Defective function of a nongenomic progesterone receptor as a sole sperm anomaly in infertile patients. *Fertil Steril* 58, 793-797.

173. C. Falsetti, E. Baldi, C. Krausz, R. Casano, P. Failli & G. Forti: Decreased responsiveness to progesterone of spermatozoa in oligozoospermic patients. *J Androl* 14, 17-22 (1993)

174. Cs. Krausz, L. Bonaccorsi, M. Luconi, B. Fuzzi, L. Criscuoli, S. Pellegrini, G. Forti & E. Baldi: Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correalted with *in vitro* fertilization. *Hum Reprod* 10, 120-124 (1995)

175. Cs. Krausz, L. Bonaccorsi, P. Maggio, M. Luconi, B. Fuzzi, L. Criscuoli, S. Pellegrini, G. Forti & E. Baldi: Two functional assays of sperm responsiveness to progesterone and their predictive values in *in vitro* fertilization. *Hum Reprod* 11 (8) (1996), in press.