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INTERACTION OF HUMAN SPERMATOZOA WITH THE ZONA PELLUCIDA OF OOCYTE: DEVELOPMENT OF THE ACROSOME REACTION

Patricio Morales[1,3] and Miguel Llanos[2]

[1]P. Catholic University of Chile, Faculty of Biological Sciences. P. O. Box 114-D and [2]INTA, University of Chile, P. O. Box 138-11. Santiago, Chile.

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1. ABSTRACT

Mammalian spermatozoa are not able to fertilize an oocyte upon ejaculation. To gain fertilizing ability, spermatozoa must, either *in vivo* or *in vitro*, undergo a process termed capacitation. Since a reliable marker for capacitation does not exist, it is considered that this process is completed when the spermatozoa are able to undergo acrosomal x

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[3] To whom correspondence should be addressed, at the Unit of Reproduction and Development, Faculty of Biological Sciences, P. Catholic University of Chile, P. O. Box 114-D, Santiago, Chile, Tel #:(562) 222-5515, Fax: (562) 222-5515, E-mail:pmorales @genes.bio.puc.cl

intestinal mucosa which might require tandem signaling events for this process is the presence of a vascular countercurrent arrangement in the subepithelial compartment (108). As happens for absorbed solutes, this countercurrent phenomenon may distort transepithelial solute gradients. For example, perfusion of mammalian intestinal loops *in vivo* with solutions containing fMLP was previously found to induce neutrophil attachment to endothelial cells and structurally defined endothelial activation, but failed to elicit directed migration across the lamina propria (Madara, unpublished observations), suggesting that directed migration may require a more stable gradient than that afforded by the usual soluble signals. For example, once present in inflamed tissue, IL-8 is likely to retain its biological activity for several hours, as shown by local intradermal administration in animals and humans (109-111). In

contrast to IL-8, chemokines such as fMLP or LTB4 are degraded rapidly by oxidation or hydrolysis (112).Fertilization is a very complex phenomenon, involving sequential interactions between the fertilizing spermatozoon and cumulus oophorus, ZP, and oolemma. The AR may be playing a key role in penetration of spermatozoa through these egg vestments.

2. INTRODUCTION

There is still debate about the composition of the human zona pellucida (ZP). SDS-PAGE of zona pellucida proteins under non-reducing conditions shows two to three main bands. The biological function of the individual components of ZP is just beginning to be recognized. Recombinant human ZP3 promotes the acrosome reaction and stimulates a protein with tyrosine kinase activity. Whether human zona pellucida or recombinant ZP3 can also stimulate sperm calcium influx is not known. Regarding the status of the sperm binding proteins for zona ligands, several candidates have been proposed. Among them are a galactosyltransferase, a D-mannosidase, a protein tyrosine kinase, and an unusual galactosyl receptor lectin. Clearly, the binding of the spermatozoa to the ZP triggers the acrosomal exocytosis. The mechanism (s) by which the acrosome reaction (AR) is stimulated by the ZP seems to be related to the production and sequential action of several intracellular messengers, with a crucial function in the development of the AR. It has been suggested that progesterone and the zona pellucida may synergistically act to promote acrosomal exocytosis in mouse. Whether the same is true in humans is not yet known; however, progesterone-treated human spermatozoa bind to the ZP in higher numbers than the non-treated spermatozoa. Other molecules have been recognized that serve a modulatory role in the spermatozoa-ZP binding process. The aim of the present review is to summarize the current knowledge about human spermatozoa-ZP interactions and the mechanisms accounting for the AR

3. THE ZONA PELLUCIDA, ITS COMPOSITION AND ROLE DURING GAMETE INTERACTION AND FERTILIZATION

All mammalian eggs are surrounded by the ZP, an extracellular coat that is synthesized by the oocyte (1). The ZP is the site of the initial interaction of the spermatozoa with the oocyte. This interaction includes the species-specific spermatozoa-ZP binding and induction of the AR, both of which are prerequisites for successful in-vivo fertilization.

Results of cDNA cloning of the ZP genes and analysis of the composition of ZP from several different species indicate that ZP is constituted of three or four glycoproteins (2). The ZP of the mouse oocyte, one of the best studied ZP, is composed of three sulphated glycoproteins termed ZP1, ZP2 and ZP3 (1, 3). ZP1 is a homodimer (Mr= 185-200 kDa) and its chains are connected by intermolecular disulphide bonds. ZP2 (Mr= 120-140 kDa) and ZP3 (Mr= 83 kDa) form a heterodimer of long filaments with a repeating structure (1, 3). ZP1 provides a structural integrity for the ZP by cross-linking the ZP2/ZP3 filaments. Only ZP2 and ZP3 have been shown to possess biological functions. ZP3 mediates the initial binding of acrosome-intact spermatozoa to the ZP via O-linked side chains (4, 5). Following sperm binding, ZP3 induces the AR in the bound spermatozoa (6). The acrosome-reacted spermatozoa, which can no longer interact with ZP3, bind to ZP2 (7) and penetrate through the ZP. After fertilization, there are molecular changes in ZP2 and ZP3 that constitute a block to polyspermy. ZP3 is converted to a form called $ZP3_f$, which no longer binds acrosome-intact spermatozoa and is incapable to induce the AR (6). Since O-linked carbohydrates are implicated in interaction of ZP3 with spermatozoa and there is no apparent change in the electrophoretic mobility of $ZP3_f$ (6, 8), this change in ZP3 is thought to be caused by a cortical granule-released glycosidase (9). ZP2 is converted to a form called $ZP2_f$ that no longer interacts with acrosome-reacted spermatozoa (10). ZP2 is cleaved by a protease from the cortical granules (11) and is detected by a shift in its electrophoretic mobility (from Mr= 120 kDa to Mr= 90 kDa) under reducing conditions (12).

In contrast to these findings in the mouse, there are only a few reports regarding the composition of the human ZP (13-17). Shabanowitz and colleagues reported only two components (Mr= 90-110 kDa and Mr= 57-73 kDa) under nonreducing conditions and three components (Mr= 90-110 kDa, Mr= 65-78 kDa, and Mr= 57-73 kDa) under reducing conditions (16, 17). They termed these proteins ZP1, ZP2 and ZP3, respectively. A ZP component corresponding to the mouse ZP1 (Mr= 200 kDa) was not detected. Similarly, under reducing conditions Bercegeay *et al.* found protein components of 80-92 kDa, 58-66 kDa, and 54-72 kDa (13). Based upon their molecular weight these components could correspond to mouse ZP2 and ZP3, respectively. Naz and Ahmad showed that the human ZP analyzed under non-reducing conditions exhibited 3 major protein bands of 220, 110 and 55 kDa. The ZP protein that reacted strongest with the sperm proteins was the 55 kDa molecular region (ZP3) (15). Recently, Moos *et al.* using a non-radioactive biotinylation- and a lectin-based detection system found that, under non-reducing conditions, the human ZP of unfertilized eggs is composed of three glycoprotein species designated as ZP1 (Mr≈150

kDa), ZP2 (Mr≈100 kDa) and ZP3 (Mr≈55-65 kDa) (14).

In all the above studies, ZP1 was not detected after fertilization. In contrast, in the mouse ZP1 is present and is apparently unaltered following fertilization (12). Therefore, it has been suggested that in humans, the cortical granule-derived proteases may degrade ZP1 to forms that are not detectable by electrophoresis (14).

4. SPERM BINDING PROTEINS/RECEPTORS FOR THE ZONA PELLUCIDA

As indicated, the glycoprotein composition of the ZP from several mammalian species has been well elucidated (18, 19). However, only limited information exists regarding the molecular identities and biochemical characteristics of the sperm surface components involved in zona binding. Several glycoprotein candidates have been proposed over the past years, among them are:

4.1. Galactosyltransferase

Galactosyltransferase is an enzyme located within the plasma membrane of spermatozoa that has a defined role in gamete recognition in the mouse (20). This enzyme mediates sperm-egg recognition by binding to N-acetylglucosamine residues on ZP glycoconjugates. Recently, the mouse zona protein ZP3 was identified as the complementary binding site for interaction with sperm β-1,4-galactosyltransferase. This interaction mediates tight spermatozoa-ZP binding (21). A potential role of multivalent binding between ZP3 and several galactosyltransferase moieties in the induction of the AR has been proposed (21). This enzyme, however, may not be present in human spermatozoa (22).

4.2. D-mannosidase

Human spermatozoa possess a D-mannosidase activity (22) which may be involved in binding to ZP3 and induction of the AR (23). The sugar moieties involved in these processes are mannose-rich oligosaccharides on zona glycoconjugates (22). A positive relationship has been demonstrated between the appearance of mannose-binding sites on the surface of human spermatozoa and the outcome of *in vitro* fertilization (24). Previous studies, however, have failed to demonstrate inhibition of spermatozoa-ZP binding by treating the spermatozoa with mannose (25, 26) (see table 1).

4.3. Protein tyrosine kinase (ZRK)

One class of molecule that could account for both ZP3 recognition and signal transduction is a receptor tyrosine kinase (27). Exposure of both mouse and human spermatozoa to ZP3 results in the rapid autophosphorylation of tyrosine residues on the putative, 95 kDa, zona receptor kinase (ZRK). Using a monoclonal antibody directed against ZRK to screen a human testicular complementary DNA library, Burks *et al*. isolated a full-length clone predicting a 600 amino acid receptor that contains a unique cysteine-rich extracellular domain (28). Peptides from the extracellular domain of this molecule suppress spermatozoa-zona binding. Recently, Saling and colleagues reported that recombinant human ZP3 ($hZP3^{rec}$), expressed in COS cells, stimulates tyrosine phosphorylation of human ZRK, *in vivo* and *in vitro* (29). Tyrphostin, a tyrosine phosphorylation inhibitor, inhibited the AR induced by $hZP3^{rec}$ (29). These results suggest that human ZRK is a receptor for human ZP3 and that ligand-stimulated tyrosine kinase activity in human spermatozoa is essential for the signaling cascade regulating exocytosis of the sperm acrosome. However, the 95 kDa phosphotyrosine-containing protein found in the mouse spermatozoa has been shown to be a unique form of phosphorylated hexokinase (30).

Naz and Ahmad reported on several sperm proteins that have the capacity to bind ZP proteins with molecular weights of 95, 63, 51 and 14-18 kDa (15). These proteins showed autophosphorylating activity. Potentially, the 95 kDa protein corresponds to the ZRK described by Saling and colleagues (28).

4.4. Lectin

An unusual galactosyl receptor, C-type (Ca^{2+}-dependent) lectin on human spermatozoa may be the zona receptor (31, 32). This lectin is a transmembrane protein with carbohydrate-recognition domains on the C-terminal extracellular segment and an N-terminal cytoplasmic anchor. This protein was characterized as a single protein component of 50 kDa confined to the plasma membrane of the head of the spermatozoa overlying the acrosome (32).

5. SPERM BINDING PROTEINS / RECEPTORS FOR THE ZONA PELLUCIDA WITH A ROLE IN THE ACROSOME REACTION

As was discussed above, work from several laboratories has revealed presence of multiple ZP3 binding proteins/receptors on the sperm cell. Therefore, it is quite possible that multiple sperm binding proteins/receptors could all be involved in mediating the biological actions of ZP3, the primary sperm binding, and induction of the AR. This multiple interactive capacity could be due to different specialized domains in ZP3, which all face the sperm head surface. Each of these domains could be structurally adequate (or specialized) to interact and/or activate a particular sperm binding protein/receptor, to permit binding of spermatozoa to the ZP3, and to subsequently induce production of

Table 1. Effect of carbohydrates on human spermatozoa binding and penetration through the human zona pellucida.

	Control*		D-mannose	
Oocyte Nº	Nº Sperm Bound	Nº Sperm Penetrating	Nº Sperm Bound	Nº Sperm Penetrating
1	17	2	4	0
2	16	15	0	0
3	16	8	9	0
4	8	3	2	0
5	16	8	9	0
6	13	8	11	0
7	3	3	2	0
8	4	1		
X±SEM	11.6±2.0	6.0±1.6	5.3±1.6	0±0

* Sperm aliquots treated for 30 min with 50 mmoles/l of D-mannose were use to inseminate single oocytes for 6 hr. Control spermatozoa were incubated with medium alone. Modified from (25 and 26).

intracellular second messengers that lead to AR. It is also possible that ZP3 interacts and activates one type of sperm receptor with the subsequent activation of other receptor (s) (33). Interaction of ZP3 with the sperm surface may occur in a multivalent fashion. Thus, different sperm binding proteins/receptors could, together, constitute a functional ZP3 receptor signal-transduction complex that is capable of transducing intracellular signals to modulate the AR (33).

In human, the candidate receptors for the ZP with a role in the AR are: a 95-kDa protein with characteristics of a protein tyrosine kinase termed zona receptor kinase (ZRK) (28) and a 51 Kd protein, that is also a tyrosine kinase and which subsequently was recognized as the human FA-1 antigen (34, 35). A role for a sperm glycine receptor/Cl^- channel in the ZP initiated AR has also recently been suggested (36).

6. INTERACTION OF SPERMATOZOA WITH THE ZONA PELLUCIDA AND DEVELOPMENT OF THE ACROSOME REACTION

ZP is involved in several essential events in fertilization of mammalian eggs. This includes species-specific sperm recognition and binding, stimulation of the sperm AR, and the egg-induced block to polyspermy. All these events are specially well documented in the mouse (1, 3). Since only acrosome-reacted spermatozoa are capable of penetrating through the ZP and fusing with the eggplasma membrane, the AR is crucial for successful fertilization of eggs in mammals (37).

The morphological characteristics of the AR in human spermatozoa are similar to that of other mammalian species investigated thus far (38). AR implies a sequential process of fusion and fenestration of the outer acrosomal membrane and its overlying plasma membrane, which permits release of soluble contents of acrosome and apparently facilitates the passage of spermatozoa through the ZP.

A subsequent formation of mixed vesicles, composed of outer acrosomal membrane and plasma membrane, occurs. These vesicles remain close to the sperm head for a short time and then disperse. Since only acrosome-reacted spermatozoa penetrate the zona, the inner acrosomal membrane and the associated hydrolytic enzymes (11, 39) together with the hyperactivated motility (37, 40) should play important functions in the penetration process.

6.1. Physiological inducers of the acrosome reaction of human spermatozoa

AR of human spermatozoa may be initiated by different molecules that can be classified as physiological or pharmacological inducers of this event. Within the pharmacological inducers, the most extensively used agents are the calcium ionophores A23187 and ionomycin. Fusogenic compounds such as lysophospholipids have also been reported to stimulate the AR in mammalian, including human spermatozoa (41-43). The preovulatory human follicular fluid (hFF; (44)) and the human ZP (45) are considered to be among the physiological stimuli for the AR in human spermatozoa. Furthermore, the human cumulus oophorus, the human mural granulosa cells and the progestins progesterone (P) and 17-α-P also stimulate AR (44, 46, 47). The AR-inducing activity of hFF and cumulus oophorus is, in part, related to their P content. Indeed, individual hFF samples exhibited great differences in their ability to induce the AR and this ability was significantly correlated with the P content of each fluid (48). Moreover, charcoal-dextran treated hFF lost the AR-initiating ability, which could be only restored after re-addition of P (48). The cumulus cells may be an additional source of P. P level in the cumulus has been estimated at 1-2 µg/ml (49), which is sufficient for induction of AR.

Extensive work has been performed to elucidate the molecules and mechanisms involved in the P-induced human sperm AR (for reviews see (50, 51)). There are some similarities between the way P and the ZP induce the AR. Effects of P on the human spermatozoa are mediated by the presence of binding proteins/receptors located on the acrosomal area of a sperm subpopulation (52, 53). P acts on the human sperm surface to induce at least three different events: i) opening of a plasma membrane Ca^{2+} channel to induce Ca^{2+} influx (54, 55). This, in turns, provokes a transient, protein kinase C (PKC)-dependent elevation of intracellular free Ca^{2+} concentration (56); ii) opening of a plasma membrane Cl^- channel to induce Cl^- efflux (57). This channel appears to be part of a receptor resembling, but no identical to, the neuronal g-aminobutyric acid receptor type A ($GABA_A$) (58); and iii) activation of a protein tyrosine kinase (59, 60). All these events seem to be due to independent actions of the steroid and are perhaps mediated by three different binding receptors (61). Thus, P may interact with a multireceptor system rather than a single receptor on the plasma membrane of spermatozoa. This hypothesis can explain the variety of responses due to nongenomic action of steroids, and reconcile experimental results that seem contradictory at the first glance (61).

The site where the fertilizing spermatozoon undergoes the AR *in vivo* is still a matter of debate. In the mouse, only acrosome-intact spermatozoa are able to bind to the ZP and, therefore, the fertilizing spermatozoon is presumed to undergo the AR on the zona surface (62). However, in several species, both acrosome-intact and acrosome-reacted spermatozoa are equally able to bind to the surface of the ZP and penetrate through it (63, 64). Acrosome-reacted human spermatozoa are also able to bind to the human ZP (65). However, it is yet not clear whether they are able to penetrate the ZP and fertilize the oocyte.

Studies on the effect of the ZP on sperm function have been carried out with intact and solubilized ZP and the purified glycoproteins, especially ZP3. More recently, some studies on human spermatozoa have demonstrated that a recombinant human ZP3 is able to promote the AR (29, 66). Also, acrosome-intact mouse spermatozoa are able to bind to ZP3 covalently linked to silica beads; such bound spermatozoa, subsequently, undergo AR and are released from the beads (67). Spermatozoa do not bind to silica beads to which a variety of other glycoproteins, including other zona glycoproteins, are covalently linked. The properties and mode of action of solubilized and purified ZP3 or recombinant ZP3, however, may not be necessarily identical to those of ZP3 immobilized on a three dimensional extracellular matrix. The ordered cross-linking of ZP2/ZP3 heterodimers by ZP1 generates the presence of stereospecific domains. These domains could be playing a role in mediating the effects of ZP3 on the sperm function. Their effects may not be apparent in studies using solubilized or purified ZP components.

7. SIGNAL TRANSDUCTION MECHANISMS AND THE ZONA PELLUCIDA-INDUCED ACROSOME REACTION

7.1. G_i proteins

A major shortcoming in the study of spermatozoa-oocyte interaction in humans is the scarcity of ZP material. Since the electrophoretic pattern of the ZP isolated from unfertilized eggs is identical to that of non-inseminated eggs, this has been overcome by the use of inseminated but unfertilized eggs (16). Using this biological material, it was demonstrated that the human ZP-induced AR

is mediated by a sperm G_i protein (68, 69). This is also true for the murine and bovine ZP-induced AR (70, 71). Thus, after the spermatozoa bind to the ZP, a G_i protein may be a crucial transducing signal. Activation of a G_i protein mediates the production of intracellular signal (s) involved in the molecular and cellular events of the AR. In cells other than spermatozoa, G_i proteins are involved in receptor-mediated regulation of: inward rectifying K^+ channels, Ca^{2+} mobilization, phospholipase C activity, and adenyl cyclase activity (72). Several of these processes have already been implicated in the mechanism that triggers the AR (73).

7.2. Intracellular free calcium

An extracellular Ca^{2+}-dependent increase in the concentration of free intracellular Ca^{2+} is usually required for the initiation of the AR. This seems to be true at least for the mouse and bovine ZP-induced AR (71, 74). Pertussis toxin inhibits the ZP/ZP3 induced elevations of intracellular Ca^{2+} (71, 75), suggesting the involvement of a G_i protein in these events. Working with bovine spermatozoa, Florman *et al.* proposed a model which explains the G_i-dependent Ca^{2+} influx promoted by ZP/ZP3 after interaction with its sperm receptor (76). Briefly, this model states that ZP/ZP3 stimulates: i) a poorly selective, G_i protein-insensitive cation channel responsible for membrane depolarization and ii) a G_i protein-dependent alkalinization of the sperm cytoplasm. These changes activate an L-type voltage-sensitive Ca^{2+} channel which, in turn, causes the massive Ca^{2+} influx necessary for the AR (76).

The relative contribution of intracellular sources of Ca^{2+}, however, has not been properly evaluated. There is increasing evidence for the presence of Ca^{2+} stores within spermatozoa which may have a role in the AR. In several somatic cells, Ca^{2+} found in the endoplasmic reticulum can be released by thapsigargin, a plant-derived sesquiterpene lactone that is a highly specific inhibitor of the Ca^{2+}-ATPase responsible for Ca^{2+} accumulation by that organelle (77, 78). Thapsigargin stimulates the AR of capacitated human (79), mouse (80), and hamster spermatozoa (81) and it requires external Ca^{2+} and influx of this ion (79, 81). Thus, the Ca^{2+} release from intracellular stores by thapsigargin may lead to an influx of extracellular Ca^{2+} and subsequently to the AR. Putative sites for thapsigargin-sensitive intracellular Ca^{2+} stores in the spermatozoa may include the cytoplasmic droplet, the sperm nucleus and the acrosome (79, 80).

Recently, the selective localization of inositol 1,4,5-trisphosphate receptors (IP_3R) in the acrosomes of rat, mouse, hamster and dog spermatozoa was reported (80). The authors also described the presence of $G_{\alpha q/11}$ and phospholipase Cβ1, and suggested a role for inositol 4,5-trisphosphate- (IP_3) gated Ca^{2+} release in the mammalian sperm AR. Walensky and Snyder presented a model where a multivalent interaction between ZP/ZP3 and sperm plasma membrane binding proteins/receptors leads to the production of multiple intracellular signals (80). Receptor activation of G_q (a pertussis toxin-insensitive process) leads to activation of phospholipase Cβ1 with the subsequent hydrolysis of phosphatidylinositol 4,5-trisphosphate and the generation of IP_3 and diacylglycerol (DAG). Then, the binding of IP_3 to IP_3R localized in the outer acrosomal membrane would induce the release of acrosomal Ca^{2+}. A subsequent capacitative Ca^{2+} entry, through focal voltage-insensitive channels, would produce a further elevation of intracytoplasmic Ca^{2+}, triggering membrane depolarization and activation of voltage-sensitive L-type Ca^{2+} channels (80). The high intracellular free Ca^{2+} concentration together with DAG production would be required for molecular events leading to membrane fusion and finally for the acrosomal exocytosis (82-84).

7.3. Polyphosphoinositides

Hydrolysis of polyphosphoinositides could be involved in the ZP-induced AR. Hydrolysis of polyphosphoinositides produces IP_3 and DAG, both of which are involved in the AR as stated above. Polyphosphoinositides are constituted by phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. In mammalian spermatozoa, polyphosphoinositides seem to be located in the inner and outer leaflets of the plasma and outer acrosomal membranes, respectively (85). In the classic model, IP_3 and DAG produced after hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phospholipase C are involved in modulation of intracellular Ca^{2+} concentration and activation of a PKC (86). In several mammalian spermatozoa, including human, the activation of PKC (a molecule able to phosphorylate proteins) is involved in the AR (87, 88). Besides the activation of PKC, DAG produced in the spermatozoa activates a phospholipase C, able to hydrolyze phosphatidyl-choline and to further increase the level of DAG (84). This high level of DAG would modulate a phospholipase A_2 (PLA_2) (82, 83). This key enzyme, which produces fusogenic compounds such as lysophospholipids and free fatty acids, is required for the development of AR (41, 42, 89).

The role of ZP in the hydrolysis of polyphosphoinositides in human spermatozoa is not known. However, in mouse spermatozoa, stimulation with ZP or P results in hydrolysis of phosphatidylinositol 4-phosphate and phosphatidyl-inositol 4,5-bisphosphate (84). Recently, it was reported that mouse ZP/ZP3 activates a phosphatidyl-

inositol 4,5-bisphosphate-PLCγ1 through a mechanism involving tyrosine phosphorylation (90). In human spermatozoa, a fraction of hFF induces hydrolysis of polyphosphoinositides that rely upon a primary Ca^{2+} entry (91). This may contradict the model proposed by Walensky and Snyder (80), in which Ca^{2+} influx occurs subsequent to and is stimulated by the mobilization of internal Ca^{2+}. Early events of the AR require micromolar Ca^{2+} levels whereas later events require millimolar levels of Ca^{2+} (82, 92). Those findings suggest that the small, rapid, and IP_3-dependent Ca^{2+} increase is due to intracellular Ca^{2+} mobilization, and that this is followed by extracellular Ca^{2+} influx.

7.4. Adenylyl cyclase activity and cAMP

Phosphodiesterase inhibitors and analogs of cAMP stimulate the AR (73). Activators of the protein kinase A pathway (PKA), such as forskolin and dibutiryl cAMP, also induce the AR of capacitated human spermatozoa (93). In addition, the hFF-induced and solubilized human ZP-induced AR are blocked by KT5720, which is an inhibitor of PKA (93). PKA inhibitors prevent the stimulation of the AR by a PKC stimulator and vice-versa (94). De Jonge suggested that a cross talk between the PKA and PKC pathways may lead to phosphorylation of proteins followed by AR (94).

An elevation of cAMP was observed during the ZP-induced mouse sperm AR (95), indicating that ZP modulates the activity of adenylyl cyclase (AC). Lecler and Kopf reported that ZP and forskolin, in a concentration dependent manner and in the presence of Mg^{2+}, but not Mn^{2+}, can stimulate a membrane associated AC in capacitated mouse spermatozoa (96). Forskolin (a stimulator of the AC) has been also reported to induce the human sperm AR, an effect that can be blocked by adenosine analogs known to inhibit AC. The latter blockage is inhibited by cAMP analogs, suggesting that forskolin acts through AC (93). Though several G proteins are present in mammalian spermatozoa, the presence of a G_s protein in these cells has not yet been documented (97). Therefore, it is not clear as how the sperm AC is activated. Further investigation is needed to elucidate the mechanism (s) by which ZP stimulates the membrane-associated AC and to reveal its role in the AR. A link between AC activation, increased cAMP concentration, Ca^{2+} channel modulation, and the development of the AR were recently suggested (96).

7.5. Membrane tyrosine kinases

Solubilized ZP stimulates a protein tyrosine kinase activity involved in the mouse sperm AR. A 95 kDa protein, found on the sperm surface, was suggested to bind ZP3, possesses tyrosine kinase activity and participates in the AR (98, 99). The role of this protein as a ZP3 receptor in the mouse, however, should be re-examined, since it was demonstrated that this protein is indeed a unique, phosphotyrosine-containing form of hexokinase, only found in the testis and spermatozoa (30). It was shown that a 95 kDa protein in the human spermatozoa interacts with human ZP (ZRK; distinct from hexokinase) and that this protein contains phosphotyrosine and tyrosine kinase activity. Moreover, recombinant human ZP3, obtained from human ZP3 cDNA transfected COS cells, stimulates kinase activity (28). In addition, in capacitated human spermatozoa, tyrphostin prevents the human ZP3-induced AR (29). Taken together, these results indicate that ZP3-induced tyrosine phosphorylation of ZRK coincides with the ZP3-induced AR, strongly suggesting a direct role for the tyrosine kinase activity in the AR of human spermatozoa. The function of tyrosine phosphorylation in the AR is still obscure. A recent report showed modulation of mouse sperm phospholipase C activity by ZP-stimulated tyrosine phosphorylation, linking the protein tyrosine kinase signaling pathway and phosphatidylinositol 4,5-bisphosphate hydrolysis (90). As discussed above, this pathway seems to play a crucial role in the AR.

8. INTRACELLULAR PROTEASES AND THE ZONA PELLUCIDA-INDUCED ACROSOME REACTION

Several investigators have proposed a role for an endogenous trypsin-like activity in the mechanism of the mammalian sperm AR (100). This trypsin-like activity may be involved in the dispersal of the acrosomal matrix, the membrane events of the AR or both (101, 102). In human, the AR induced by the human ZP and FF is inhibited by several trypsin inhibitors (103). These results suggest that hFF and human ZP induce the human sperm AR through a common mechanism mediated by a trypsin-like protease activity. A chymotrypsin-like activity is also involved in the human ZP- and hFF-induced sperm AR (100). The precise role of this chymotrypsin-like activity, however, remains to be elucidated. In sea urchin spermatozoa, a chymotrypsin-like enzyme is involved in the activation of Ca^{2+} channels and leads to an increase in the level of intracellular Ca^{2+}, a crucial requisite for the AR (104). The hFF-induced AR also induces a rapid and transient increase in the level of intracellular Ca^{2+} due to Ca^{2+} influx (54, 55). This event can be inhibited by trypsin and chymotrypsin inhibitors (105, 106), suggesting a role for both types of protease activity in the elevation of intracellular Ca^{2+} level. In the mouse and bovine spermatozoa, the ZP-induced AR is associated with a Ca^{2+} influx (71, 74). Therefore, it could be speculated that the effect of trypsin and chymotrypsin inhibitors on the human ZP-induced AR (100, 103) may be due to an inhibition of the ZP-stimulated Ca^{2+} influx before the induction of the AR. Further research is required to clarify the identity and possible role of

these trypsin and chymotrypsin-like activities in the human sperm AR.

9. CELLULAR BIOLOGY OF HUMAN SPERMATOZOA-ZONA PELLUCIDA INTERACTION

In response to an oocyte-derived signal or signals, at the time of fertilization of the oocyte, the spermatozoon undergoes acrosomal exocytosis (37). The generally accepted view is that the fertilizing spermatozoon traverses the cumulus oophorus using the hyaluronidase activity present in the protein PH20, located on the plasma membrane of the posterior head (107). Then, the fertilizing spermatozoon, with its acrosome-intact, binds to the zona pellucida glycoprotein, ZP3. Following stimulation by ZP3, the spermatozoon undergoes the AR. Exposure to ZP3 leads to tyrosine phosphorylation of a 95 kDa protein (15, 28, 29) and activation of a guanosine triphosphate (GTP)-binding protein (68). Progesterone (P) trapped in or produced by the cumulus oophorus can also initiate the AR (46). P action is specific (54, 58), is mediated by a surface receptor (108), and leads to phosphorylation of a 95 kDa protein (60). G protein activation, however, does not take place (109).

9.1. Modulators of spermatozoa-zona pellucida binding

9.1.1. Progesterone

Interaction between P and the ZP has been reported in the mouse (110). During sperm activation, both agonists sequentially interact. As compared with simultaneous presence of both P and ZP or presence of agonists in the reverse order, spermatozoa treated sequentially with half maximal concentrations of P and ZP exhibited enhanced exocytosis (110). Based on these findings, it was concluded that P exerts a priming effect in the initiation of the AR. Whether a similar interaction between P and ZP takes place during human fertilization is not known. Regarding spermatozoa-ZP binding, P may (111) or may not (112) increase the total number of spermatozoa able to bind to the ZP. Recent experiments conducted in our laboratory have shown that P definitively increases the number of zona bound spermatozoa. Treatment of the spermatozoa with P (1 µg/ml) caused a significant increased (292±25%) in their capacity to bind to the ZP. This effect required presence of calcium in the culture medium (Morales and Llanos, unpublished results).

9.1.2. Fucoidin

Fucoidin is a heteropolisaccharide consisting primarily of α(1'2)- and β(1'3)-linked L-fucose sulfate subunits. Treatment of the spermatozoa with fucoidin inhibits spermatozoa-ZP binding in humans (113). The specificity of the fucoidin, however, is ambiguous since incubation of ZP with fucoidin also caused inhibition of spermatozoa-zona binding (113).

9.1.3. Sialic acid

Removal of sialic acid may also play an important role in spermatozoa-zona binding since treatment of the spermatozoa with neuraminidase, an enzyme that cleaves α(2⌧3)- or α(2⌧8)-linked sialic acid residues, enhanced attachment of spermatozoa to the human ZP (114). Removal of sialic acid from the sperm surface is associated with loss of net negative charges (115) and sperm capacitation (116).

9.1.4. D-Mannose

Previous studies have attributed a role to the sperm mannose-binding sites on the spermatozoa-ZP binding process (24-26, 117). However, Mori and colleagues observed only a 50% decrease in the number of spermatozoa bound to the ZP even at the highest mannose concentration tested (50 mmol/l) (25, 26). Zona penetration was completely inhibited (table I). A 30% inhibition of spermatozoa-ZP binding by mannose was observed by Oehninger *et al.* (118). Chen *et al.* found that treatment of the spermatozoa with mannose caused a 70% inhibition of spermatozoa-ZP binding (117). Inhibition of binding, however, was not observed during the initial phase of the spermatozoa-ZP interaction. Acrosin is one of the candidates to support binding of acrosome-reacted spermatozoa to ZP (119, 120). The action of human acrosin is inhibited by a variety of monosaccharides, especially D-fructose and D-mannose (121). Based on these observations, the data presented above can be reinterpret as follows. Mannose-treated spermatozoa can initiate binding to the human ZP followed by development of AR in the spermatozoa bound to the zona surface. Since acrosin activity is inhibited after AR, spermatozoa cannot perforate a path through the zona and such spermatozoa eventually detach from ZP.

9.1.5. Egg yolk and Milk

Egg yolk and milk were recently suggested to increase the number of spermatozoa that bind to the human ZP (122, 123). The mechanism of action and the biological significance of these observations are not yet clear.

9.1.6. Placental protein 14

Recently, it was reported that human placental protein 14, a glycoprotein synthesized and secreted by the endometrial cells, significantly reduced spermatozoa-zona binding (124). This effect was specific for this protein and not for other endometrial stromal products. Since placental protein 14 is also expressed in ectopic endometrium (125),

the authors suggested that this may provide a mechanism for endometriosis-related infertility (124).

9.1.7. Gonadotropin hormone-releasing hormone (GnRH)

Some recent reports have indicated that GnRH and analogs (agonists and antagonists) may modulate spermatozoa-ZP binding in humans (126, 127). GnRH is a decapeptide of hypothalamic origin which causes the release of LH and FSH from the pituitary. GnRH or GnRH-like molecules have been detected in hFF (128) and human seminal plasma (129, 130). These molecules are probably synthesized in the gonads (131-134) and in the prostate (135).

Treatment of the spermatozoa with 20 nM GnRH for 5 min increased, by 357±14%, the number of zona bound spermatozoa in comparison to the control, saline treated spermatozoa (126). Busereline, a GnRH agonist, increased the binding by 326±32%. Treatment of the spermatozoa with the GnRH antagonist 4pF (20 nM) did not cause any significant change in the number of zona-bound spermatozoa. However, 4pF administered 5 min before the addition of GnRH completely blocked the stimulatory effect of GnRH. Treatment of the spermatozoa with GnRH does not modify the percentage of acrosome reactions or the pattern of sperm movement (126).

The above findings suggest that the spermatozoa may interact with GnRH, or GnRH-like molecules at different steps during the reproductive process: 1) during spermatogenesis by local, intratesticular production (136-139); 2) during sperm maturation in the epididymis; 3) during ejaculation, upon mixing with seminal plasma (129, 130, 135); and 4) during the ascent to the site of fertilization in the ampulla of the oviduct. In the oviduct, the spermatozoa may interact with GnRH secreted locally or transported by the products of ovulation (follicular fluid, granulose cells) from the ovary (128, 131, 133).

10. CONCLUDING REMARKS

There are still many unanswered questions regarding mammalian sperm interaction with the oocyte. The requirement for sperm capacitation as well as the acrosomal status of the spermatozoa traveling through the cumulus oophorus needs to be studied further. Although efforts have been made to establish the characteristics of the proteins present in the sperm surface which act as the receptor for ZP3 glycoprotein, the exact function for these proteins in the mechanism associated to the ZP3-induced AR has not yet been elucidated. Thus, future studies should focus on determining the exact nature of these receptors, their structure, and function. The presence of neuronal receptors (such as GABA-like and glycine receptor) in mammalian spermatozoa with a role in the AR, either induced by progesterone or solubilized ZP, has been recently suggested. In addition, the AR, a major regulatory event for mammalian spermatozoa-oocyte interaction leading to fertilization, requires further studies to integrate the information regarding the role of the different signaling pathways. These studies may provide the precise sequential role of the intracellular messengers produced after the spermatozoa-ZP interaction has taken place. If previously reacted spermatozoa can initiate binding to the ZP, one major question is whether they are able to penetrate the ZP and fertilize the oocyte. The nature of the zona (ZP2) receptor in the acrosomal-reacted spermatozoa and the acrosomal proteases in the development of the AR and penetration through the ZP need to be clarified. Investigation of these and other related questions should unravel the molecular processes involved in the acrosome reaction.

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