

Kinases, phosphatases and proteases during sperm capacitation

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Abstract Fertilization is the process by which male and female haploid gametes (sperm and egg) unite to produce a genetically distinct individual. In mammals, fertilization involves a number of sequential steps, including sperm migration through the female genital tract, sperm penetration through the cumulus mass, sperm adhesion and binding to the zona pellucida, acrosome exocytosis, sperm penetration through the zona and fusion of the sperm and egg plasma membranes. However, freshly ejaculated sperm are not capable of fertilizing an oocyte. They must first undergo a series of biochemical and physiological changes, collectively known as capacitation, before acquiring fertilizing capabilities. Several molecules are required for successful capacitation and *in vitro* fertilization; these include bicarbonate, serum albumin (normally bovine serum albumin, BSA) and Ca^{2+} . Bicarbonate activates the sperm protein soluble adenylyl cyclase (SACY), which results in increased levels of cAMP and cAMP-dependent protein kinase (PKA) activation. The response to bicarbonate is fast and cAMP levels increase within 60 s followed by an increase in PKA activity. Several studies with an anti-phospho-PKA substrate antibody have demonstrated a rapid increase in protein phosphorylation in human, mouse and boar sperm. The target proteins of PKA are not known and the precise role of BSA during capacitation is unclear. Most of the studies provide support for the idea that BSA acts by removing cholesterol from the sperm. The loss of cholesterol has been

suggested to affect the bilayer of the sperm plasma membrane making it more fusogenic. The relationship between cholesterol loss and the activation of the cAMP/PKA pathway is also unclear. During early stages of capacitation, Ca^{2+} might be involved in the stimulation of SACY, although definitive proof is lacking. Protein tyrosine phosphorylation is another landmark of capacitation but occurs during the late stages of capacitation on a different time-scale from cAMP/PKA activation. Additionally, the tyrosine kinases present in sperm are not well characterized. Although protein phosphorylation depends upon the balanced action of protein kinases and protein phosphatase, we have even less information regarding the role of protein phosphatases during sperm capacitation. Over the last few years, several reports have pointed out that the ubiquitin-proteasome system might play a role during sperm capacitation, acrosome reaction and/or sperm-egg fusion. In the present review, we summarize the information regarding the role of protein kinases, phosphatases and the proteasome during sperm capacitation. Where appropriate, we give examples of the way that these molecules interact and regulate each other's activities.

Keywords Sperm · Capacitation · Kinases · Phosphatases · Proteasome

Introduction

As the world's population continues to grow at an alarming speed (<http://www.census.gov/ipc/www/popclockworld.html>), which threatens the ecological balance of the planet, there is an increasing need to develop new methods of birth control and explore the molecular bases of fertilization (Nass and Strauss 2004). Fertilization is the result of an ordered series of cellular

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interactions and understanding the underlying mechanisms of fertilization can provide insight into basic cellular processes. Fertilization is the process by which two haploid gametes, namely the sperm and egg, unite to produce a genetically distinct individual. In mammals, fertilization involves a number of sequential steps, including sperm migration through the female genital tract, sperm penetration through the cumulus mass, sperm adhesion and binding to the zona pellucida (ZP), acrosome exocytosis, sperm penetration through the ZP and fusion of the sperm and egg plasma membranes (Yanagimachi 1994). However, freshly ejaculated sperm are not capable of fertilizing an oocyte. They must first undergo a cascade of biochemical and physiological changes that facilitate the binding and penetration of the sperm into the oocyte. This time-dependent acquisition of fertilization competence has been defined as "capacitation" (Yanagimachi 1994).

Capacitation

Capacitation was discovered independently in 1951 by Austin (1951) and Chang (1951) in rats and rabbits, respectively. They observed that mammalian sperm must reside in the female reproductive tract for a period of time in order to attain fertilizing capacity. Capacitation confers upon the sperm the ability to gain hyperactive motility, interact with the ZP, undergo the acrosome reaction (AR) and initiate oocyte plasma membrane fusion (Yanagimachi 1994). Capacitation normally occurs in the female genital tract; however, it can also be achieved *in vitro*. The discovery that certain factors in the female genital tract were needed for the sperm to become fertile was a necessary step for the consequent development of *in vitro* fertilization. In 2010, the British physiologist Robert Edwards, whose work led to the first "test-tube baby," won the Nobel Prize in Medicine for the development of *in vitro* fertilization, a breakthrough that has helped millions of infertile couples worldwide to have children.

Capacitation has been defined as a combination of sequential and parallel processes that occur both in the head (preparation for the AR) and sperm tail (hyperactivation). Recently, capacitation has been divided into: (1) fast and early events that comprise activation of the vigorous and asymmetric movement of the flagella and that happen as soon as the sperm leave the epididymis and (2) slow and late events that comprise changes in the pattern of movement (hyperactivation), the ability to carry out the AR stimulated by a physiological agonist and the phosphorylation of tyrosine in proteins (Salicioni et al. 2007; Visconti 2009). *In vivo*, these events take place during the passage of sperm within the female reproductive tract. They can also take place by incubating the sperm in an appropriate culture medium.

In the next section, we briefly describe these processes. Although some controversy exists about whether the initial rapid events are part of capacitation, they are essential for the events that take place later and seem to be necessary for the successful achievement of fertilization. The rapid events occur a few seconds after the sperm are ejaculated, based on the high concentrations of Ca^{2+} and HCO_3^- present in the seminal fluid (Visconti 2009). The HCO_3^- enters the sperm through the cotransporter $\text{Na}^+/\text{HCO}_3^-$ (Demarco et al. 2003). This increase in the HCO_3^- concentration produces an increase in the intracellular pH and the activation of a unique type of adenylyl cyclase present in the sperm, the soluble adenylyl cyclase (SACY). The main characteristic of SACY is that is activated by HCO_3^- and Ca^{2+} and not by protein G or forskolin, as are most of the transmembrane adenylyl cyclases. In turn, the physiological levels of HCO_3^- produce a rapid collapse of the asymmetry of the sperm plasma membrane attributable to the activation of scramblase enzymes that translocate membrane phospholipids, such as phosphatidylserine and phosphatidylethanolamine (Gadella and Harrison 2000), increasing the availability of cholesterol to external acceptors (Salicioni et al. 2007; Visconti 2009). At the same time, SACY increases the intracellular levels of cAMP and, subsequently, the activation of protein kinase A (PKA). The activation of PKA modulates the response of calcium channels such as CatSper, which produces changes in the membrane potential (Wennemuth et al. 2003) and increases in the intracellular Ca^{2+} concentration (Fig. 1).

The beginning of the slow events of capacitation is marked by the removal of cholesterol from the membrane by BSA and the increase in its fluidity. BSA can be substituted in *in vitro* capacitation media by cholesterol-binding compounds such as cyclodextrins (Visconti et al. 1999). This allows the sperm to maintain high levels of HCO_3^- . In addition, during capacitation, the cholesterol efflux content of the sperm membranes decreases and lipid-raft domains reorganize (Cross 2004). During this phase, PKA phosphorylates several proteins in Ser and Thr residues, activating, either directly or indirectly, several protein kinases and/or inhibiting protein phosphatases, which will finally produce an increase in the phosphorylation of Tyr residues. Eventually, all these changes will lead to the capacitation of the sperm and as a final point, the following events: (1) the ability to carry out the AR induced by biological agonist, ZP, or progesterone; (2) hyperactivation; (3) chemotaxis, although this is controversial in mammals, as only capacitated sperm are thought to exhibit chemotactic behavior (Salicioni et al. 2007); and (4) the ability to fertilize an oocyte.

The paradox of the regulation of late and fast capacitation events is that they both seem to be modulated by the same molecules, namely HCO_3^- , SACY and cAMP (Salicioni et

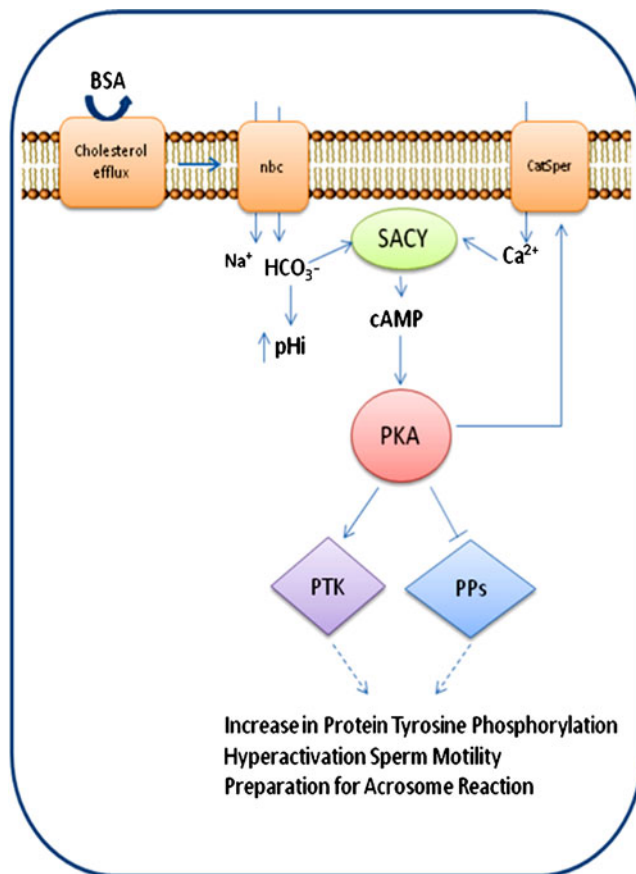


Fig. 1 Molecular basis of events associated with capacitation. Once ejaculated, the Ca²⁺ and HCO₃⁻ present in the seminal fluid enter the sperm through calcium channels (e.g., *Catsper*) and a Na⁺/HCO₃⁻ cotransporter (*nbc*), respectively. These ions activate soluble adenylyl cyclase (*SACY*), which activates protein kinase A (*PKA*), which, through the activation of protein tyrosine kinases and/or the inhibition of protein phosphatases, allows the activation of flagellar movement. The beginning of slow events seems to be initiated by the elimination of cholesterol from the sperm membrane. The maintenance of high levels of Ca²⁺ and HCO₃⁻ keep *SACY* active, which activates *PKA*, which, through the activation of tyrosine kinases or the inhibition of phosphatases, allows an increase in Tyr phosphorylation, hyperactivation and acrosomal exocytosis. All these events are necessary for the sperm to acquire fertilizing capacity (*BSA* bovine serum albumin, *PTK* non-receptor tyrosine kinases, *PPs* protein phosphatases, *pHi* internal pH)

al. 2007; Visconti 2009). Evidence promoting this hypothesis has been presented by Esposito et al. (2004), Harrison (2004), Harrison and Miller (2000) and Hess et al. (2005). The study of Morgan and colleagues (2008) has provided clear evidence that the same pathways are activated both in the rapid and late events. Morgan et al. (2008) have worked with mice that bear a mutation in the catalytic subunit, the ATP-binding site, of *PKA*. This mutation allows the specific binding, in this modified site, of the pyrazolo[3,4-d]pyrimidine inhibitor, INM-PP1, without affecting the activity of other kinases. With this inhibitor, Morgan et al. (2008) have blocked, in the mutant mice, the increase in the HCO₃⁻-dependent flagellar frequency, the phosphorylation of

PKA substrates that occurs within 90 s of HCO₃⁻ addition and the increase in Tyr phosphorylation; the authors conclude that *PKA* has at least two independent roles in the regulation of sperm movement: a quick action that is required for the activation of the flagellar beat and a slow action that allows changes in the pattern of movement of the sperm and that requires that *PKA* remains active for an extended period of time (Visconti 2009).

Protein phosphorylation and kinases involved in capacitation

Because mature sperm are transcriptionally quiescent, protein phosphorylation is an invaluable method of controlling their physiology (for reviews, see Naz and Rajesh 2004; Urner and Sakkas 2003). The rapid activation of *PKA* during sperm capacitation in vitro has been discussed above. Naz et al. (1991) and Visconti et al. (1995) have reported that the Tyr phosphorylation state is enhanced in an array of human and mouse sperm proteins during capacitation in vitro and that this process is dependent upon *PKA*. A similar pathway, which is unique to sperm, has been subsequently reported for numerous species (for a review, see Bailey 2010).

Tyr protein phosphorylation

The identification of proteins undergoing Tyr phosphorylation during capacitation is, at the moment, incomplete. So far, the identified Tyr-phosphorylated proteins in human sperm include ion channels; metabolic enzymes and structural proteins (Ficarro et al. 2003); *CABYR*, a calcium-binding protein localized in the principal piece of the tail in association with the fibrous sheath (Naaby-Hansen et al. 2002); and members of the ERK (extracellular-signal-regulated kinase) family (Luconi et al. 1998a, 1998b). The main Tyr-phosphorylated structural proteins of the fibrous sheath (Ficarro et al. 2003) are the family of A-kinase-anchoring proteins (AKAPs) and their involvement in motility has been defined (Muratori et al. 2011).

Tyr protein phosphorylation of human sperm does not require Ca²⁺ in the extracellular medium (Muratori et al. 2011), whereas *BSA* and HCO₃⁻ are required. A decrease in membrane cholesterol content appears to promote the phenomenon (Shadan et al. 2004) and a high cholesterol content in the membrane might underlie a defect in phosphorylation in subfertile/infertile men (Buffone et al. 2009). Despite having demonstrated that the process is regulated by a cAMP-dependent pathway (Visconti et al. 1995), the involvement of *PKA* upstream of the process is not clear (see below) and the downstream tyrosine kinase(s) (TK), which

link(s) the cAMP pathway to the increase in Tyr phosphorylation remain(s) elusive. Evidence in murine (Baker et al. 2006) and bovine (Lalancette et al. 2006) sperm pointed to c-src (cellular-sarcoma) as being one of the kinases that might play this role. In human sperm, c-src inhibition has been shown to decrease Tyr phosphorylation during capacitation (Varano et al. 2008). However, as recently demonstrated, c-src-KO mice undergo the capacitation normally, including the increases in Tyr phosphorylation (Krapf et al. 2010), ruling out a key involvement of the kinase in the process.

An increase in the Tyr phosphorylation of sperm proteins is generally recognized as a signature of late-stage capacitation (Arcelay et al. 2008; Visconti et al. 1995). Using a chemical genetic approach, the McKnight group have demonstrated that acute PKA inhibition blocks this late increase in Tyr phosphorylation and the phosphorylation of PKA substrates occurring within 90 s of the addition of HCO_3^- to sperm (Morgan et al. 2008). Late (30 min) PKA inhibition still blocks the normal induction of Tyr phosphorylation. This result suggests that PKA activity must be sustained for capacitation to proceed normally (Morgan et al. 2008).

Whatever the role of Tyr phosphorylation in capacitation, the level of Tyr phosphorylation in human sperm correlates strongly with the sperm-zona-binding capacity (Liu et al. 2006) and alterations in Tyr phosphorylation have been found in subfertile subjects (Buffone et al. 2009) indicating its physiological role in fertilization.

Tyrosine kinases

As mentioned above, an increase in Tyr-phosphorylated proteins is essential for capacitation. This increase in Tyr phosphorylation is carried out by the activation of a group of enzymes, the TKs. This group of proteins is turned on, either directly or indirectly, by PKA. In addition, the TKs can be divided into two classes called receptor TKs (RTKs) and non-receptor TKs (PTKs). The RTKs are transmembrane proteins with an extracellular binding domain to a ligand and an intracellular TK domain; in contrast, the PTKs are located in the cytoplasm, nucleus, or the inner side of the plasma membrane. PI3-kinase activity (a non-receptor TK) has been detected in human sperm (Fisher et al. 1998, Tomes et al. 1996).

The presence of other TKs (PTKs and RTKs) has been described in sperm of several species, such as c-ras in human sperm (Naz et al. 1992), the receptor TK epidermal growth factor in sperm of rabbit, mouse, rat, human (Naz and Rajesh 2004) and bovine (Lax et al. 1994) and c-Abl in human (Naz 1998) and mouse (Baker et al. 2009) sperm. In mouse sperm, Baker et al. 2009 have shown, *in vitro*, that

PKA activates c-Abl through phosphorylation of Thr residues; *in vivo*, they have determined that PKA is associated with c-Abl and that PKA phosphorylate c-Abl in the tail of the sperm.

Recently, several groups have demonstrated the involvement of PTKs, such as the members of the Src family (SRC, FYN, LYN and YES1) in sperm capacitation in several species. The PTK c-yes (cellular-yamaguchi sarcoma viral oncogene) has been detected in the head of human sperm and its activity is dependent on cAMP. However, no cAMP-dependent phosphorylation sites are present in its molecular structure, so that its mechanism of action has not yet been elucidated (Leclerc and Goupil 2002). SRC, LYN and FYN are found mainly in the tail of human sperm.

SRC is also present in the sperm head but its active form (pY416) is located in the middle piece of human sperm. SRC has been one of the most studied TKs in recent years, since it has been identified in human sperm (Varano et al. 2008). It has sites that are phosphorylated by PKA and it co-immunoprecipitates with PKA. SRC becomes activated by phosphorylation of the Y416 residue during human sperm capacitation. Furthermore, the suppression of PKA and SRC through the use of specific inhibitors leads to a dramatic decrease in Tyr phosphorylation. However, although the inhibition of PKA is also accompanied by a suppression of sperm motility, SRC inhibition does not induce a similar response (Mitchell et al. 2008). SRC interferes with the response to calcium triggered by progesterone (Varano et al. 2008). SRC has also been described in mouse sperm (Baker et al. 2006; Krapf et al. 2010). In mouse sperm, it is located mainly in the flagella and is associated with hyperactivation induced by cAMP analogs (Baker et al. 2006). In addition, Kraft and collaborators have found that SRC family inhibitors block *in vitro* fertilization in the mouse, reinforcing the idea that SRC is a mediator of capacitation (Krapf et al. 2010). All of these studies suggest that TKs are involved in the increase in Tyr phosphorylation that occurs during capacitation. However, as PKA is a Ser/Thr kinase, the phosphorylation of these residues (Ser and Thr) must also fulfill an important role during capacitation.

Ser/Thr kinases

PKA is a ubiquitous tetrameric enzyme that contains two regulatory subunits and two catalytic subunits; its activity depends on cAMP. The binding of cAMP to the regulatory subunits allows tetramer dissociation and the activation of the catalytic subunit (Nolan et al. 2004). In human and mouse sperm, PKA is located on the acrosomal region and in the flagellum (Pariset and Weinman 1994; Visconti et al. 1995). Sperm PKA has a unique catalytic subunit (i.e., $\text{C}\alpha 2$). Mice lacking the catalytic domain produce a normal

number of sperm that swim spontaneously in vitro. However, $C\alpha 2$ -null mice sperm do not exhibit an increase in the pattern of Tyr phosphorylation after incubation under capacitating conditions (see below; Esposito et al. 2004; Hess et al. 2005) and are infertile despite normal mating behavior (Nolan et al. 2004). Additionally, two inhibitors of PKA have been reported to inhibit protein Tyr phosphorylation and sperm capacitation (Visconti et al. 1995). Collectively, these data demonstrate that this kinase has an important role in sperm function.

Protein kinase C (PKC) is also present in mammalian sperm, possibly with a role in sperm movement and the AR (Breitbart and Naor 1999).

Ser/Thr protein phosphorylation

In spite of the numerous reports of Tyr phosphorylation, only a few studies have been published on the phosphorylation of Ser/Thr residues reflecting the difficulties in performing such studies. One of the first works to describe phosphorylation on Ser or Thr residues was that of Naz in 1999. In this work, at least four groups of protein were determined as being phosphorylated on Ser; their molecular masses were: 43–55 kDa, 94 kDa, 110 kDa and 190 kDa. In addition, lighter bands of 18 and 35 kDa were observed. The Thr-phosphorylated proteins were within the same range of sizes. In both cases, the phosphorylation increased during capacitation and after exposure to the ZP. In addition, some of these proteins were demonstrated also to be phosphorylated at Tyr residues. Subsequently, Beddu-Addo et al. (2005) assessed the phosphorylation on Ser, Thr and Tyr of two groups of proteins of 105 kDa and 81 kDa (Leclerc et al. 1996) and determined that these phosphorylations were dependent on the incubation time, the phosphorylation on Ser/Thr being an earlier event than the phosphorylation on Tyr. In addition, these phosphorylation events were reversible and dependent on the presence of BSA and HCO_3^- . To assess the role of HCO_3^- and/or BSA in the phosphorylation of the PKA motif, sperm were incubated in media deficient in one or both of these components. As expected, the sperm incubated in the absence of both HCO_3^- and BSA showed extremely low levels of phosphorylation. In humans, the presence of BSA or HCO_3^- alone seemed to be sufficient to increase the Ser/Thr phosphorylation of sperm proteins to levels similar to those in complete medium (Bedu-Addo et al. 2005). However, the data were obtained after 6 h of capacitation, i.e., considerably later than the cAMP-PKA-stimulated phosphorylation seen within minutes of capacitation. Mouse sperm do not exhibit Ser/Thr phosphorylation in the absence of HCO_3^- and the role of BSA is not clear in this species. In addition, PKA activity in capacitating mouse sperm is higher in the presence of BSA than in its absence

(Visconti et al. 1999). In boar sperm, HCO_3^- -induced Ser/Thr phosphorylation is markedly reduced in the absence of BSA. Moreover, after HCO_3^- stimulation in the absence of BSA, the levels of cellular cAMP are significantly lower than after stimulation in its presence (Harrison 2004). On the other hand, we (Kong et al. 2009) have determined that, during capacitation, several proteins phosphorylated on their Ser and Thr residues have molecular weights between 27–134 kDa and 23–124 kDa, respectively and that the degree of phosphorylation of these proteins increases during capacitation. However, epoxomicin, a proteasome inhibitor, prevents the increase in phosphorylation on Ser and decreases the phosphorylation on Thr of proteins of 115 and 124 kDa. However, no changes are seen in the Tyr-phosphorylated proteins. These results suggest that the proteasome is involved in the Ser and Thr phosphorylation that takes place during capacitation but not in Tyr phosphorylation.

In pig sperm, a large group of proteins are phosphorylated on their Ser/Thr residues during capacitation (Harayama and Nakamura 2008). This phosphorylation presents a specific kinetic, reaching a peak after 80 s, then decreasing and increasing again slowly until the end of the incubation period (Harayama and Nakamura 2008; Harrison 2004). In the presence of Calyculin A (CL-A), a Ser/Thr phosphatase inhibitor, phosphorylation in these residues increases markedly (Harrison 2004). Contrary to this work, the group of Alnagar (2010) using antibodies that recognize the phosphorylation of Ser/Thr residues within a consensus sequence present in some kinase substrates (Akt, PKA and PKC) have noted a dephosphorylation of five phosphorylated proteins in Ser/Thr (96, 90, 64 and 55 kDa) within 15 min of capacitation in the pig. In addition, using CL-A during sperm capacitation, Alnagar et al. (2010) have determined that dephosphorylation does not take place. These results provide evidence that the dynamics of phosphorylation on Ser and Thr residues is more complex than is normally described during capacitation.

Protein phosphatases

Currently, a consensus has been reached that the regulation of cellular processes requires the coordinated action between kinases and phosphatases. Our knowledge about the protein kinases involved in these processes is broad but less extensive work and studies have been carried out on the participation of protein phosphatases (PPs) in cellular processes. According to the amino acidic residue that is dephosphorylated, PPs are classified into the following groups: protein tyrosine phosphatases (PTP), which dephosphorylate amino acids at Tyr residues and protein Ser/Thr phosphatases (PSP), which dephosphorylate amino acids at Ser and/or Thr residues. The human genome project has found

518 putative PKs. The high specificity of the signal and the nature of reversible phosphorylation suggest that the number of PPs should be similar. However, only 107 PTP have been detected (Alonso et al. 2004) and even fewer PSP, i.e., approximately 30. Whereas the number of TKs and PTP is similar, the number of catalytic subunits of PSP is an order of magnitude lower than the number of serine kinases. This dichotomy can be explained because PSP are holoenzymes that are made from a combination of subunits, in which the catalytic subunit is shared and associated with a large number of regulatory subunits (Shi 2009).

Protein tyrosine phosphatases

Protein tyrosine phosphatases are encoded by a large family of phosphatase genes. These enzymes are defined by the active-site signature motif HCX5R, in which the cysteine residue functions as a nucleophile and is essential for catalysis. These enzymes are divided into the classical phosphotyrosine (pTyr)-specific phosphatases, dual-specificity phosphatases (DSPs) and low-molecular-weight PTP (Stoker 2005; Tonks 2006). The classical PTPs include transmembrane receptor-like proteins (RTPs) and non-transmembrane cytoplasmic PTPs. RTPs are predominantly associated with the plasma membrane and have the potential to regulate signaling through ligand-activated dephosphorylation. Many of these phosphatases have been implicated in the processes of cellular adhesion and communication. The non-transmembrane cytoplasmic PTPs do not have transmembrane segments and are located in various subcellular compartments, such as the cytosol, plasma membrane and the endoplasmic reticulum. They have regulatory sequences near the catalytic site allowing them to control their activity through protein-protein interactions (SH2 domains) or to control the specificity of substrate. Some of these phosphatases act as positive and negative regulators of cell adhesion and motility (Zhang et al. 2004).

The DSPs are enzymes less well conserved, have little sequence similarity beyond the cysteine-containing signature motif and possess smaller catalytic domains than the classical PTPs. The construction of the DSP active site allows them to accommodate phosphoserine (pSer)/phosphothreonine (pThr) residues and pTyr residues in proteins. The DSPs include the VH1-like enzymes (which are related to the prototypic VH1 DSP, a 20-kDa protein that is a virulence factor of vaccine virus) and the mitogen-activated protein kinase (MAPK) phosphatases (MKPs). MKPs are the best characterized sub-divisions of these enzymes and catalyze the inactivation of MAPKs by the dephosphorylation of both Tyr and Thr phosphorylation sites in the kinase activation loop. These MKPs display distinct patterns of induction, subcellular location and specificity for

individual MAPKs, thereby representing a response network of phosphatases that function in attenuating MAPK-dependent signaling pathways (Dickinson and Keyse 2006; Kondoh and Nishida 2007; Tonks 2006).

A protein similar to VH1, VHY (VH1-related member Y), has been reported in the testis and, through immunohistochemical analysis, has been detected in pachytene spermatocytes and spermatids but is poorly observable in Leydig cell and has not been seen in mature sperm (Alonso et al. 2004; Nakamura et al. 1999). Various studies have shown that the MKPs have an important role in the regulation of immune function, stress response and metabolic homeostasis (Dickinson and Keyse 2006; Kondoh and Nishida 2007; Tonks 2006). MKP-1 and MKP-3 PPs are degraded by the ubiquitin-proteasome pathway (Choi et al. 2006; Marchetti et al. 2005; see also below) and thus this pathway might degrade any of the PPs present in the sperm.

Serine/threonine phosphatases

Before the mid 1980s, little was known about the relationship between the many phosphatases described in the literature. Then, in 1983, Ingebritsen and Cohen (1983) suggested that most of the activities of PPs described in the literature could be explained by the activity of only four principal catalytic subunits (designated as PP1, PP2A, PP2B and PP2C) with broad and overlapping substrate specificities. Today, we know that the actual situation is more complex. In the naming system pioneered by Ingebritsen and Cohen (1983), the known PPs were categorized based on (1) their biochemical characteristics; (2) their sensitivity to endogenous inhibitor proteins; (3) their differential sensitivity to specific inhibitors; and (4) a limited amount of substrate specificity that could be demonstrated *in vitro*. In this system, the mammalian phosphatases are placed into two major subtypes (PP1, PP2; for excellent reviews, see Cohen 1989; Cohen et al. 1990; Hunter 1995). The catalytic subunit of PP1 preferentially dephosphorylates the β -subunit of phosphorylase kinase and its activity toward phosphorylase kinase is inhibited by two heat-stable cytosolic proteins referred to as inhibitor-1 and inhibitor-2 (I1 and I2). The PP2 family preferentially dephosphorylates the α -subunit of phosphorylase kinase and its members are not sensitive to inhibition by I1 or I2. PP2 is further divided into three subtypes that are distinguished by their requirements for divalent cations. The subtype designated PP2A is active in the absence of divalent cations and is inhibited by nanomolar concentration of okadaic acid (OA) and microcystin (Cohen 1989), whereas the subtypes designated as PP2B (calcineurin) and PP2C require Ca^{2+} /calmodulin and Mg^{2+} , respectively (Honkanen and Golden 2002). In addition,

PP2B is much less responsive to OA and microcystin and PP2C is resistant to these inhibitors.

On the basis of the information obtained from the human genome sequencing project, we have been able to complete the classification proposed by Ingebritsen and Cohen (1983). A comparison of the primary sequence of the PPs has revealed that PP1, PP2A and PP2B are related, whereas PP2C is structurally different and belongs to a different family of genes (for a review, see Cohen 1997). Within the Ser/Thr-specific PPs and after comparing their primary sequence, three distinct gene families have been described: the PPM (Mg^{2+} - or Mn^{2+} -dependent PPs), the FCP (transcription-factor-IIF-associating C-terminal domain phosphatases) and the PPP (phosphoprotein phosphatases; Table 1). The PPM family comprises the Mg^{2+} -dependent PPs, such as pyruvate dehydrogenase, PP2C and relatives (Barford et al. 1998). The FCP family comprises FCP1 and small C-terminal domain phosphatases 1–3 (Gallego and Virshup 2005; Yeo et al. 2003). The PPP family includes PP1, PP2A/PP4/PP6, PP2B, PP5 and PP7 gene subfamilies that share high homology in the catalytic domains but differ in their N- and C-terminal domains (Barford et al. 1998; Cohen 1997; Fardilha et al. 2011; Honkanen and Golden 2002).

Phosphoprotein phosphatases

In most of the members of the PPP family, the catalytic subunit is associated with a variety of regulatory subunits. This has made us think that these PPs are promiscuous and,

therefore, not interesting to study. However, in the cell, PSP are forced multimeric enzymes that are created by the assembly of a small number of catalytic subunits combined with hundreds of regulatory subunits. The combinatorial and regulatory complexity that occurs in these PPs produces high accuracy (Virshup and Shenolikar 2009), generating enzymes that can decipher environmental signals and that can control and co-coordinate highly regulated biochemical events. The PPPs bind to a variety of substrates through their own structural motives. They have various cellular localizations and participate in the regulation of many cellular functions. In somatic cells, PP1 is involved in the regulation of the metabolism, cell cycle, cellular signaling and muscle contraction (Cohen 1989, 2002). PP2A participates in the regulation of the cell cycle, keeping the M-phase-promoting factor inactive and Cdc25 (cell division cycle 25) active (Janssens and Goris 2001; Lee et al. 1991; Yamashita et al. 1990). In addition, PP2A modulates the activity of protein kinases such as PKA, PKC, ERK/MAPK (Liauw and Steinberg 1996; Millward et al. 1999) and participates in the beginning and ending of translation, in apoptosis and in the response to stress (Janssens and Goris 2001; Santoro et al. 1998). PP2B, also known as calcineurin, is a calcium-dependent PP that is responsible for the transcription of interleukin-2 to stimulate growth and T-cell differentiation and that regulates some of the cell cycle proteins, among other functions. PP4 and PP6 are structurally related to PP2A (Becker et al. 1994; Brewis et al. 1993).

Distinct genes encode PP4 and PP6 and when their primary sequence was compared with that of PP2A, they were found to have 65% and 57% homology, respectively

Table 1 Classification of Ser/Thr phosphatases (PPP phosphoprotein phosphatase, PPM Mg^{2+} - or Mn^{2+} -dependent protein phosphatases, FCP transcription-factor-IIF-associating C-terminal domain phosphatases, MC microcystin, OA okadaic acid, CL-A Calyculin A, AKAPs A-

kinase-anchoring proteins, IGBP1 immunoglobulin-binding protein 1, SET myeloid-leukemia-associated protein, CIP2A cancerous inhibitor of protein phosphatase 2A). Modified from Fardilha et al. (2011)

Family	Subfamily	Genes	Catalytic subunits	Regulatory subunits	Subunit composition	Selected inhibitors
PPP	PP1(PPP1)	3	α , β , γ 1, γ 2	>100 PIPs	Heterodimer and higher order	Inhibitor-1, Inhibitor-2, MC, OA, CL-A
	PP2A(PPP2)	2	α , β	A subunit, family of B subunit (B, B', B'', B''')	Heterodimer and higher order	SET, CIP2A, Fostriecin, OA, MC, CL-A, Nodularin, Endothal
	PP4 (PPPA)	1	1	IGBP1, PR4R1, PPP4R2, PR4R3 β , c-Rel	Heterodimer and higher order	OA, MC, CL-A, Tautomycin
	PP6 (PPP6)	1	1	IGBP1	Heterodimer and higher order	OA, MC, CL-A
	PP2B (PPP3)	3	3	Calcineurin B, AKAPs, Calmodulin	Heterodimer and higher order	Fenvalerato, Deltamethrin, Ciclosporine, FK506
	PP5 (PPP5)	1	1		Monomer and higher order	OA, MC, CL-A, Nodularin
	PP7 (PPP7)	2	2	Calmodulin	Monomer	Inorganic phosphate
PPM	PP2C	16	>22 isoforms	None	Monomer	?
FCP	FCP1	1	4		Monomer	?

(Huang et al. 1997). PP4 has been found in the centrosomes of mammals and *Drosophila* embryos (Sumiyoshi et al. 2002). PP4 also has a proapoptotic role in T-cells (Mourtada-Maarabouni et al. 2003) and interacts with components of nuclear factor kappa B (c-Rel, p50, RelA; Hu et al. 1998; Zhou et al. 2004). PP6 has been detected in lysates of human heart, muscle and bull testis. In human cells, the highest levels of expression of mRNA have been found in testis, heart and skeletal muscle (Bastians and Ponstingl 1996).

Recently, Kajino et al. (2006) have shown that PP6 is a negative regulator of TAK1 (transforming-growth-factor-activated kinase 1), a Ser/Thr kinase member of the family of MAPKKK and essential component of intracellular signaling in the inflammatory signaling pathway. PP5 (Becker et al. 1994; Cohen 1997) and PP7 (Huang and Honkanen 1998) are also part of the family of the PPPs. Both contain a catalytic domain common to the family of the PPs: PP1/PP2A/PP4. However, they differ in the size of their N-terminal and C-terminal domains, which are involved in the accuracy and regulation of their enzymatic activity. In humans, a single gene exists for PP5 and two genes codify PP7 (Honkanen and Golden 2002). PP5 is present in all mammalian cells but the level varies between tissues, presenting enhanced expression in the brain. Its subcellular localization is both nuclear and cytoplasmic. Studies have shown that it is a regulator of the signaling pathway of the glucocorticoids receptor. On the other hand, PP5 has a role in the cell cycle by negatively regulating p53. PP7 is a PP isolated from *Arabidopsis*. In humans, its expression is

restricted to the fetal retina, during brain development and in primary sensitive neurons (Honkanen and Golden 2002). In addition, it has been found to bind to calmodulin in a calcium-dependent manner and is located in the nucleus of cells (Moller et al. 2003).

Sperm phosphatases

Sperm capacitation is a process highly regulated by kinases. However, little is known about the participation of PPs in this process. Strong evidence has been presented that certain PPs have an important role during sperm maturation (Chakrabarti et al. 2007a; Mishra et al. 2003; Vijayaraghavan et al. 1996) allowing the sperm to acquire movement. Additionally, once sperm are ejaculated, the PPs also have a role in the acquisition of hyperactivated motility (Fardilha et al. 2011; Hoskins et al. 1983; Krapf et al. 2010; Smith et al. 1996). However, little information is available about the participation of PPs during capacitation. This is in spite of the findings that, when PKA become active during capacitation, it phosphorylates the Ser/Thr residues of proteins and that the regulation of the phosphorylated state of those proteins requires the participation of PSP.

Table 2 shows the expression of the PPP family of PSP and those that are present in testis and sperm.

Early work on sperm PPs appeared at the end of the 1980s by the group of Tash (Tash and Bracho 1994; Tash et al. 1988; Table 2). They identified and characterized

Table 2 Expression of the family PPP of PSP in various tissues and in testis and sperm (ND not determined) modified from Fardilha et al. (2011)

Phosphatase	Other tissues	Testis	Sperm
PP1 α	Ubiquitous; more predominantly in brain and lung (Shima et al. 1993a)	Yes (Shima et al. 1993b)	Yes (Suzuki et al. 2010; Fardilha et al. 2011)
PP1 β	Ubiquitous; more predominantly in brain, intestine and lung (Shima et al. 1993a)	Yes (Shima et al. 1993b)	No
PP1 γ 1	Ubiquitous; more predominantly in brain (Shima et al. 1993a)	Yes (Shima et al. 1993b)	No
PP1 γ 2	Low abundance (Strack et al. 1999)	Yes (Kitagawa et al. 1990; Sasaki et al. 1990; Shima et al. 1993b)	Yes (Smith et al. 1996; Shima et al. 1993b; Vijayaraghavan et al. 1996)
PP2A	Ubiquitous; more predominantly in brain (Khever-Goodall and Hemmings 1988)	Yes (Kloeker et al. 2003)	Yes (Tash et al. 1988; P. Morales, unpublished data)
PP2B	Ubiquitous; more predominantly in brain (Wallace et al. 1980)	Yes (Ueki et al. 1992; Chang et al. 1994)	Yes (Vijayaraghavan et al. 1996; Huang et al. 2005; P. Morales, unpublished data)
PP4	Ubiquitous; more predominantly in lung, liver and kidney (Hu et al. 2001)	Yes, highly abundant (Hu et al. 2001; Kloeker et al. 2003)	ND
PP5	Ubiquitous; more predominantly brain (Becker et al. 1994; Chinkers 1994)	Yes (Becker et al. 1994)	ND
PP6	Ubiquitous; more predominantly heart and skeletal muscle (Bastians and Ponstingl 1996)	Yes, highly abundant (Bastians and Ponstingl 1996; Kloeker et al. 2003)	ND
PP7	Ubiquitous; more predominantly sensory organs (Andreeva and Kutuzov 2009)	Yes, highly abundant (Andreeva and Kutuzov 2009)	ND

PP2B in sperm of dog, boar and sea urchin, determining its presence in the postacrosomal region and tail of the sperm. Functionally, they showed that PP2B has a role in the acquisition of sperm movement. In 1996, the enzymatic activity of some PSPs was established (as classified by Ingebritsen and Cohen 1983) in sperm extracts of sea urchin (Tash et al. 1988), cattle (Tang and Hoskins 1975), goat (Barua et al. 1985), dog, pig (Tash et al. 1988) and human (Ahmad et al. 1995). The expression of PP1 α (Ashizawa et al. 1994) and PP2A (Ashizawa et al. 2006) was shown by Western blot of fowl sperm. Using specific inhibitors for PP1, PP2A, and PP2B, Ashizawa et al. (1994, 2004) showed that these PPs were involved in the AR but that PP1 rather than PP2A played an important role in regulating fowl sperm movement at 40°C.

In mammals, four isoforms of the catalytic subunit of PP1 have been found and are encoded by the following individual genes: PP1 α , PP1 β , PP1 γ 1 and PP1 γ 2 (Chakrabarti et al. 2007b). PP1 γ 2 appears to be the main PP1 isoform in sperm (Smith et al. 1996) and is important in regulating sperm motility (Mishra et al. 2003; Smith et al. 1996). The PP1 γ 2 isoform has been detected in mouse, hamster, bull, primate and human sperm (Chakrabarti et al. 2007b; Han et al. 2007; Smith et al. 1996) and co-localizes with AKAP220 in cytoskeleton structures (Reinton et al. 2000).

PP2B, the calcium/calmodulin-dependent phosphatase, or calcineurin, has been detected in dog, goat, porcine, mouse and bovine sperm (Carrera et al. 1996; Tash et al. 1988). PP2A has been detected in bovine (Vijayaraghavan et al. 1996) and fowl (Ashizawa et al. 2006) sperm. The role of PP2B and PP2A in sperm physiology is still unclear. We have evidence indicating the presence of PP2B and PP2A in human sperm and their possible involvement in sperm capacitation (Signorelli et al. 2011).

Mouse

The first work in this species was carried out by Muramatsu et al. (1992), based on the work of Tash (see above). They cloned PP2B from cDNA obtained from mouse testis. The same year, Furuya and colleagues (1992) evaluated the effect of PSP inhibitors, OA and CL-A, during the capacitation of mouse sperm. Capacitation was evaluated by using the chlortetracycline (CTC) fluorescent assay. They found that incubation with these inhibitors induced a rapid increase (≤ 15 min) in capacitation. Subsequently, after 2–3 h of incubation, the percentage capacitated sperm was the same in control and treated sperm. This was the first work that presented a direct observation of the relationship between PSP and capacitation. Currently, by using a mouse knockout for the PP1 gene, Chakrabarti et al. (2007a, 2007b) have observed that these mice are infertile because they have a disrupted spermatogenesis. The cellular localization of some of the PP1 isoforms that are expressed in mouse sperm has been evaluated. Thus,

PP1 γ 2 is principally located in the cytoplasm of secondary spermatocytes, round and elongated spermatids and testicular and epididymal sperm. However, the expression of PP1 γ 2 is weak or absent in spermatogonia, pachytene spermatocytes and interstitial cells in which PP1 γ 1 and PP1 α are predominantly expressed (Chakrabarti et al. 2007b). Soler and colleagues (2009) have rescued the knockout phenotype of PP1 by expressing PP1 γ 2 transgenically in these mice. Unexpectedly, the expression of transgenic PP1 γ 2 restores the development and viability of spermatids and spermiation but does not restore the normal ultrastructure of the sperm tail. The evidence suggests that PP1 γ 2 is essential for the germ cell but is not enough for the process of normal spermatogenesis. The correct expression in space and time is required for the two isoforms of PP1 to produce a healthy sperm with a normal structure during spermatogenesis.

In 2009, Goto and Harayama evaluated the phosphorylation of PKA substrates in the presence of CL-A (PP1 and PP2A inhibitor) and cBIMPS (cAMP analog). They showed that the phosphorylation pattern of these proteins increases during capacitation, with proteins of 250, 170, 155, 140 and 42 kDa. In addition, they noted that these proteins are located in the mid and principal pieces. Functionally, by inhibiting PP1 and PP2A, the progressive motility decreased and the percentage of hyperactivated sperm increased. When only PP1 was inhibited with CL-A, an increase occurred in the active form of PKA (PKA phosphorylated in Thr-197) and the inactive form of PP1 (PP1 phosphorylated in Thr-320). All these results suggest that the PPs sensitive to CL-A inhibit the activation of PKA and the increase of phosphorylation of flagellar proteins in a way that prevents the early changes in the sperm movement, from progressive movement to hyperactivation (Goto and Harayama 2009).

Finally, the group of Visconti (Krapf et al. 2010) has revealed that Src inhibition is also able to inhibit PKA phosphorylation, sperm motility and in vitro fertilization and that these effects are overcome when sperm are incubated in the presence of Ser/Thr phosphatase inhibitors such as OA and CL-A at concentrations reported to affect only PP2A. With these results, the authors propose a parallel pathway by which the Src kinase can be regulated by the activity of Ser/Thr PPs, particularly PP2A. These PPs present a consensus site of phosphorylation (TPDYFL) in their C-terminal domain of the catalytic subunit, which when phosphorylated, inhibits their activity (Krapf et al. 2010). If this path is active in sperm, the inhibition of SRC might occur when PP2A is active and might lead to the dephosphorylation of Ser/Thr substrates (Krapf et al. 2010, Visconti et al. 2011).

Bovine

With regard to the sperm of this species, the group of Vijayaraghavan has performed the most meaningful studies

and has contributed much knowledge to this topic. In 1996, Vijayaraghavan and colleagues (Table 2), working with bovine sperm from the upper and lower sections of the epididymis, reported the presence of PP1 γ 1, PP1 γ 2, PP2 α (less) and GSK-3 protein (glycogen synthase kinase-3, endogenous modulator of PP1) and the activity of PP1 γ 1, PP1 γ 2 and GSK-3. In addition, they demonstrated that, even though PP1 α , PP1 β and PP1 γ 1 are ubiquitously expressed, PP1 γ 2 is expressed as in testis and seems to be the only isoform present in sperm. This has been evaluated in various mammal species (mouse, hamster, cattle, monkeys and human). However, in *Xenopus* sperm, the isoform PP1 γ 1 and not the isoform PP1 γ 2 is present, suggesting that PP1 γ 2 is present only in mammalian sperm. Functionally, Vijayaraghavan et al. (1996) evaluated motility from epididymal sperm in the presence of PP inhibitors (primarily of PP1). They observed that movement of immotile sperm from the head of the epididymis can be induced and that the kinetic activity of mature sperm from the tail of the epididymis can be stimulated. These results showed that the PPs, in particular PP1, had an important role in sperm maturation. Additionally, they identified several endogenous modulators of PP1 activity, such as the sds22 protein (Huang et al. 2002; Mishra et al. 2003), the 14-3-3 protein (Aitken 2006; Puri et al. 2008) and I2 (Vijayaraghavan et al. 1996, 2000) and I3 (Chakrabarti et al. 2007a; summarized by Fardilha et al. 2011).

Pig

The studies of Harayama have shown that, in the presence of a cAMP analog (cBIMPS), the rate of capacitated sperm, as evaluated by CTC assay, increases after 90 and 180 min of incubation and that when CL-A is added, no change occurs in this pattern (Harayama 2003; Harayama and Nakamura 2008). Non-capacitated sperm incubated with cBIMPS present Ser/Thr-phosphorylated proteins (PKA substrates) in the postacrosomal region and when capacitation increases, postacrosomal phosphorylation diminishes or disappears and the fluorescence of these proteins increases toward the tail (Harayama 2003). However, when CL-A is added to capacitated sperm, the fluorescence in the postacrosomal region and the tail is maintained. Adachi et al. (2008) have evaluated the presence of the inactive form of PP1 (phosphorylated PP1) by immunofluorescence and found that it is located in the postacrosomal region of non-capacitated sperm; these authors have also evaluated the effect of cBIMPS and CL-A during the AR and noted that cBIMPS increases the rate of AR sperm (approximately 50%) but that, when CL-A is added, this increase disappears (Adachi et al. 2008). These results suggest that PP1 is present in the postacrosomal region and participates in the dephosphorylation of the proteins of this region, independently of

capacitation time. However, pig sperm requires this process of dephosphorylation to undergo the AR. This indicates that postacrosomal phosphoproteins have a role in the suppression of premature AR before and after ejaculation.

Hamster

In this model, Si and Okuno (1999) and Leclerc et al. (1998) evaluated the hyperactivation and Tyr phosphorylation in the presence of CL-A. Their results suggested that this inhibitor promoted hyperactivation and Tyr phosphorylation of a protein of 80 kDa. No other study of PPs of hamster sperm then appeared, until recently, when Suzuki et al. (2010; Table 2) examined the regulation of hyperactivation by PP2A. First, they determined the presence of PP1 γ , PP1 α , PP2B (low in sperm) and the catalytic subunit of PP2A in hamster sperm. Subsequently, they investigated the role of these PPs on hyperactivation by using three different inhibitors of PP1 and PP2A (OA, CL-A and tautomycin) at various concentrations. They noted that none of the three inhibitors influenced the progressive motility of the sperm but that sperm hyperactivation increased in treated sperm compared with the control without inhibitor (Suzuki et al. 2010), as Si and Okuno (1999) had reported. However, the PP2B inhibitors deltamethrin and fenvalerate at various concentrations produced no changes. When PP1 α is Thr-phosphorylated and PP2A is Tyr-phosphorylated, both PPs are known to become inactive. Suzuki et al. (2010) evaluated the activity of these two PPs by using specific antibodies. Their results showed that PP1 α became phosphorylated in the first 2 h (inactive during the activation of motility and sperm hyperactivation) but became dephosphorylated after 3 h of incubation (active in hyperactivated sperm). On the other hand, PP2A was not phosphorylated at the beginning of capacitation, when it was active but after 30 min of incubation and up to 4 h, it was phosphorylated, indicating that it was inactive during hyperactivation acquisition and in hyperactivated sperm (Suzuki et al. 2010). Finally, they evaluated whether the two proteins of 80 and 85 kDa were Tyr-phosphorylated in the presence of various concentrations of OA or CL-A at various times of incubation. They observed that the Tyr phosphorylation of both protein increased with time, although this increase was not associated with hyperactivation when PP2A was inhibited but was associated with hyperactivation when both PP1 and PP2A were inhibited. Although they studied the PP1 isoform PP1 α , they did not discard the participation of PP1 γ in this process.

Human

The first work on human sperm in this regard was performed by Furuya et al. in 1993. They evaluated sperm incubated with CL-A and observed a rapid increase (≤ 15 min) in capacitated sperm. This effect was maintained until it was

similar to that of the control group after 2 to 3 h. A couple of years later, Ahmad et al. (1995) identified the expression of PP2B and evaluated its role in human sperm motility and hyperactivation. They found that PP2B regulated sperm movement parameters and that both motility and hyperactivation were dependent on calmodulin. In 1996, Smith et al. showed the presence of PP1 γ 1, PP1 γ 2 and GSK-3 and evaluated the participation of PP1 in the motility of human sperm. They observed that, in the presence of PP inhibitors, the movement parameters increased. The same year, Leclerc et al. (1996) used OA to inhibit PP2A and CL-A to inhibit PP1 and then evaluated movement parameters, the Tyr phosphorylation of p105/81 proteins and capacitation. This study showed that CL-A increased sperm velocity, the level of phosphotyrosine proteins and the rate of capacitated sperm (like Furuya et al. 1993). However, OA reduced the lateral displacement of the head, caused no changes in Tyr phosphorylation and produced an increase in the percent of capacitated sperm. These results suggest that PP1 is involved in sperm motility and capacitation but that PP2A only participates in capacitation.

Phosphorylation and proteasomes

The phosphorylation of many proteins is known to act as a signal that triggers their degradation by the ubiquitin-proteasome system (UPS; for reviews, see Ciechanover 1998; Glickman and Ciechanover 2002; Rubin and Finley 1995; Tanaka and Chiba 1998). Other cellular models have shown that phosphatases are targeted by this system (Brush and Shenolikar 2008; Trockenbacher et al. 2001). Our cellular model (spermatozoa) reveals two reasons that the relationship between protein phosphorylation and proteasomal activity is of interest: (1) because proteasomes have been shown to be present in mammalian sperm and to play a significant role during the fertilization process (Morales et al. 2003, 2004, 2007; Sutovsky et al. 2000, 2004; Tipler et al. 1997); (2) because the phosphorylation of some of the subunits is an important mechanism in the functional regulation of the proteasome (Bose et al. 1999; Mason et al. 1996, 1998; Pardo et al. 1998; Rivett et al. 2001).

The ubiquitin-proteasome system

The 2004 Nobel Prize in Chemistry was awarded to a group of scientists for the discovery of the degradation of proteins mediated by UPS (Ciechanover 2005b; Hershko 2005; Rose 2005). UPS degrades most long- and short-lived normal and abnormal intracellular proteins (Goldberg 2003). In the UPS, most substrates are first marked for degradation by covalent linkages to multiple ubiquitin molecules. Ubiquitin,

an evolutionarily highly conserved 76-amino-acid protein, is covalently linked to proteins in a multistep process involving the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) enzymes. Poly-ubiquitin chains are assembled via an isopeptidic linkage between a Lys residue of the previous ubiquitin and the C-terminal Gly residue of the subsequent ubiquitin. Because of the presence of seven Lys residues in the ubiquitin molecule, various multi-ubiquitin chains can be formed (Bedford et al. 2010). Chains of four or more ubiquitin moieties linked via Lys48 of ubiquitin are known to represent the usual signal for proteasome-mediated proteolysis (Hicke and Dunn 2003). The process of ubiquitination is balanced by the process of de-ubiquitination, which is mediated by a number of enzymes. Once marked by poly-ubiquitin chains, proteins are rapidly degraded by the 26S proteasome.

Recent studies have indicated that the dysregulation of UPS is profoundly involved in several human diseases (for a review, see Zhang et al. 2007b) and UPS has emerged as a potential therapeutic target for their treatment (Nalepa et al. 2006). Recently, UPS has been implicated in male infertility. Compared with sperm donor control samples, asthenozoospermic samples exhibit a higher amount of the proteasome β 3 subunit (Martinez-Heredia et al. 2008) and lower amounts of the proteasome α 3 subunit (Siva et al. 2010).

The proteasome

The proteasome is responsible for the majority of protein degradation in the cell. Proteolytically active sites reside within the 20S core particle (CP), which is a cylindrically shaped structure formed by four stacked rings in a α 7 β 7 β 7 α 7 pattern (Ciechanover 1998, 2005a, 2006). Subunits β 2, β 5, and β 1 have proteolytic activity and display trypsin-like, chymotrypsin-like and caspase-like peptidase activity, respectively (Groll et al. 2001). Whereas the CP can degrade some proteins, most degradation depends on ATP. The only ATP-hydrolyzing proteins in the proteasome are the six ATPases present in the regulatory particle (RP; Bedford et al. 2010; Gallastegui and Groll 2010). The 19 subunits of the RP are divided between two substructures: the base and lid complexes. The lid contains the Rpn11 subunit, which has deubiquitinating activity. The base consists of a ring increase AAA-ATPases. The CP can associate with one or two RPs but other activators might also bind to the 20S CP (for reviews, see Bedford et al. 2010; Gallastegui and Groll 2010).

Regulation of the proteasome

A major finding regarding proteasome regulation is that 26S proteasome function is stimulated by PKA phosphorylation

in mammals (Zhang et al. 2007a). Phosphorylation of the ATPase subunits, Ser120 of RPT6 in particular, by PKA correlates with increased chymotryptic and tryptic activity and is reversible by treatment with PP1 γ (Zhang et al. 2007a). Ping's group (Zong et al. 2006) has shown that cardiac 20S proteasomes display distinct patterns of endogenous Ser/Thr phosphorylation and that these phosphorylation patterns are modulated by PP2A and PKA, which coexist within the 20S proteasome complex. Furthermore, PKA activity or the inhibition of PP2A significantly augments the proteolytic function of cardiac proteasomes (Zong et al. 2006). Multiple individual subunits of the 20S complex (e.g., α 1 and β 2) appear to be the targets of PP2A and PKA (Zong et al. 2006). Shao et al. (2010) have also reported that proteasome activity is enhanced by 8-bromo-cAMP, forskolin and activators of PKA. As has been previously discussed, PKA, PP1 γ and PP2A are present in human sperm. Whether they form complexes with the sperm proteasome is not known. Asai and colleagues (2009) have provided unequivocal evidence that PKA enhances the assembly and activity of cardiac 26S proteasomes, both in vitro and in vivo. In addition, proteasomes immunoprecipitated by a monoclonal antibody demonstrate significantly lower activities when also dephosphorylated by acid phosphatase (Mason et al. 1996).

All of the evidence supports the concept that phosphorylation serves as a key regulatory mechanism of proteasome function and emphasizes the role of PKA and PPs in proteasome regulation. It also provides additional support for our hypothesis, which states that the cAMP/PKA pathway regulates proteasome phosphorylation and activity in human sperm.

UPS during fertilization

Proteasomes are present in sperm from numerous species and their involvement in several sperm functions, including capacitation, has been addressed (for a review, see Zimmerman and Sutovsky 2009).

Marine invertebrate sperm

In invertebrate sperm, the proteasome is involved in multiple stages of the fertilization process, from the AR triggered by the egg jelly to the penetration of the vitelline membrane and fusion with the egg plasma membrane (for a review, see Sakai et al. 2004). Briefly, in the solitary ascidian, *Halocynthia roretzi*, the sperm proteasome is necessary for binding to and penetration/digestion through the vitelline coat (Saitoh et al. 1993; Sawada et al. 1983; Yokosawa et al. 1987). In the ascidian, *Ciona intestinalis*, the sperm proteasome is involved in binding (Marino et al. 1992;

Pinto et al. 1990) and penetration (Sawada et al. 1998) through the egg vitelline membrane. In the sea urchin, fertilization is inhibited by proteasome inhibitors (Yokota and Sawada 2007). Moreover, proteasome inhibitors block egg-jelly-induced calcium influx, AR (Matsumura and Aketa 1991) and sperm penetration through the vitelline coat (Yokota and Sawada 2007).

Mammalian sperm

Several studies have shown that mammalian sperm have all of the components of the 26S proteasome (human: Baker et al. 2007; Morales et al. 2003; Tipler et al. 1997; Wojcik et al. 2000; rat: Baker et al. 2008; Haraguchi et al. 2007; Pizarro et al. 2004; mouse: Baker et al. 2008; Pasten et al. 2005; Pizarro et al. 2004). Additionally, multiple ubiquitin-specific proteases (Baker et al. 2008) and ubiquitin-conjugating enzymes E2 (Fischer et al. 2005; Sutovsky et al. 2000; Tipler et al. 1997) and E3 (Rivkin et al. 2009; Rodriguez and Stewart 2007; Wong et al. 2002) have been identified in the proteomes of mammalian sperm. In mouse and human sperm, the proteasome subunit α 6 has been detected as a substrate for Tyr phosphorylation during capacitation (Arcelay et al. 2008; Ficarro et al. 2003). Haraguchi et al. (2007) have shown the presence of ubiquitinated proteins in the nucleus of rat and human sperm. However, little information is available on the role of the sperm proteasome during mammalian fertilization. In the pig, Peter Sutovsky's group have observed the inhibition of sperm penetration through the ZP with proteasome inhibitors and antibodies, without inhibition of the AR (Sutovsky et al. 2004). Recently, they have presented evidence that the sperm acrosome-born 26S proteasomes recognize and degrade ubiquitinated ZP proteins during pig fertilization (Zimmerman et al. 2011). Notably, sperm proteasomes are excluded from mature sperm chromatin during spermatogenesis and the failure of this process might be associated with sperm abnormalities (Bialy et al. 2001; Ziemba et al. 2002).

Over the last few years, our group has gathered information on the role of the sperm proteasome in mammalian fertilization. The information can be summarized as follows. In humans, the sperm proteasome is involved in the exocytosis of the acrosome and in the sustained phase of the Ca²⁺ influx that precedes it (Morales et al. 2003). Sperm treatment with proteasome inhibitors blocks capacitation and changes the pattern of protein phosphorylation without affecting sperm motility or the ZP-binding capacity (Kong et al. 2009). Moreover, TK and PKA inhibitors decrease the activity of the proteasome during capacitation. Immunoprecipitation and Western blot analysis have indicated that proteasomes are phosphorylated during capacitation in a TK- and PKA-dependent pathway and that this

phosphorylation is responsible for the increase in proteasome activity (Kong et al. 2009; Morales et al. 2007). These findings support the notion that sperm proteasomes have an active role in the capacitation process and that proteasome activity is modulated by protein kinases. We have also shown that an extracellular pool of proteasomes is located on the plasma membrane of the sperm head (Morales et al. 2004). This has been confirmed for pig (Yi et al. 2007), mouse (Pasten et al. 2005) and ascidian (Sawada et al. 2002) sperm. A function for these extracellular proteasomes has been provided in the pig by Peter Sutovsky's group (Yi et al. 2007; Zimmerman et al. 2011). We have also shown that proteasome phosphorylation and activity is regulated by the extracellular matrix proteins fibronectin and laminin (Diaz et al. 2007; Tapia et al. 2011). This observation supports the idea that the cumulus cells and their matrix play an important role in fertilization (Jin et al. 2011). In relation to the proteasome and male fertility, we have presented evidence that human sperm with defective centriolar/pericentriolar structures have decreased proteasomal activity and that poor quality sperm display an intrinsic proteasome activity deficiency, which might be associated with their low fertilizing potential (Rawe et al. 2008; Rosales et al. 2011). These observations support the involvement of the sperm proteasome in male infertility. Finally, we have validated the existence of proteasomes in sperm from several mammalian species (Pizarro et al. 2004) and demonstrated their importance during in vitro fertilization and acrosomal exocytosis in mouse and cattle (Pasten et al. 2005; Sanchez et al. 2011).

Concluding remarks

Taking together, all the evidence given in this brief review of the literature suggest that, during the process of sperm capacitation in mammals, protein kinases and phosphatases play a balanced role, regulating the phosphorylation state of sperm proteins. Although much work has been performed in the field of sperm kinases, sound evidence indicates that phosphates also play a significant role. Recently, the importance of the sperm proteasome has started to be recognized during the fertilization process. However, we are still short of studies that point out the relationship, if any, between kinases, phosphatases and the UPS in the regulation of sperm physiology.

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