Chapter 7
Boar Spermatozoa Within the Oviductal Environment (II): Sperm Capacitation

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Abstract Around ovulation, a set of changes leads to a destabilisation of the sperm membrane that results in the release of spermatozoa from the oviductal reservoir (see Chap. 6). This destabilisation of the sperm membrane is an early step of the capacitation process, is mediated by bicarbonate, and allows AQN-1 to be shed from the surface. After being released from sperm reservoir, spermatozoa freely swim from the isthmus towards the ampulla/ampullary–isthmic junction where, amongst others, the following crucial and sequential events take place: (1) completion of sperm capacitation, (2) binding of spermatozoa to the ZP of the oocyte, (3) acrosome exocytosis and (4) further membrane fusion. The present chapter deals with the first issue, and thus focuses on the changes that the spermatozoon undergoes during capacitation. These changes, which can be separated between early/fast and late/slow, entail the activation of several signalling pathways, the increase of certain intracellular messengers, such as Ca^{2+} and cAMP, the reorganisation of proteins and lipids of sperm plasmalemma, and changes in motility patterns. Finally, destabilisation of the acrosomal sperm head membrane increases the sperm's ability to bind the zona pellucida of the oocyte.

7.1 Introduction

Uncapacitated and freshly ejaculated spermatozoa are not able to interact with ZP and fuse with the oocyte, but they need to undergo a prior priming sequence of events (Visconti 2009). This physiological process, known as sperm

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capacitation, was first discovered in 1951 in independent observations that Austin and Chang made in rats and rabbits, respectively (Austin 1951; Chang 1951, 1959). Indeed, both authors found that spermatozoa must reside in the female reproductive tract for a period of time prior to gaining fertilising ability (Signorelli et al. 2012).

We can define sperm capacitation as a complex and lengthy physiological process that involves a combination of sequential and parallel molecular changes that affect both the sperm head and the sperm tail (Suarez 2007). This process allows spermatozoa to acquire the ability to fertilise the oocyte, and mainly takes place when the spermatozoa are in the vicinity of the oocytes at the ampullary–isthmic junction (Rodríguez-Martínez et al. 2005; Rodríguez-Martínez 2007; Tulsiani et al. 2007). In fact, there are three sequential events that take place while the spermatozoa become capacitating/capacitated (Sutovsky 2009, 2011). All these events occur within the oviduct:

1. Detachment from the oviductal sperm reservoir (see also Chap. 6),
2. Remodelling of the acrosome surface in a required step prior to acrosome exocytosis mediated by ZP, and
3. Priming of sperm membranes for fusion with the oolemma.

It is worth noting that the ability of spermatozoa to bind to intact-ZP, only lasts for a relatively short period (from hours to days) and this depends on the species. In addition, the time required for spermatozoa to 'switch-on' and become fertile is species specific, being shorter for some species and longer for others (Fraser 2010). In the case of boar, capacitation of spermatozoa takes about 2 h on average to be completed (Botto et al. 2010). Some authors believe that events taking place immediately after ejaculation and before reaching the oviduct should also be considered as part of sperm capacitation, since they are required steps prior to the events occurring within the oviduct (Visconti 2009). This aspect will be taken up again when dealing with fast capacitation events.

Finally, we must mention that even though many biochemical and cell biological indicators of capacitation have been described, the sequence of steps to achieve full capacitation (i.e. the ability to interact immediately with the oocyte) has yet to be defined (Visconti 2009; Fraser 2010). However, it is quite evident that the ability of spermatozoa to bind ZP involves considerable remodelling of sperm plasmalemma, since, as mentioned before, uncapacitated spermatozoa are not able to recognise the oocyte and do not show innate fusibility.

7.2 A General Overview of the Changes that Sperm Undergo During Capacitation

During capacitation, spermatozoa undergo a wide array of changes and at the end of this process, i.e. when a spermatozoon is fully capacitated, the apical plasma membrane of the sperm head has become fusogenic (Harrison 1996, 2004).

Summarising, the most important are (Töpfer-Petersen et al. 2002; Tardif et al. 2003):

– First remodelling of plasma membrane architecture and cholesterol-efflux that increases membrane fluidity and provokes lipid scrambling and increases membrane fluidity (Harrison et al. 1996; Gadella and Harrison 2002; Harrison and Gadella 2005).
– Up-regulation of cellular signalling pathways (Kalab et al. 1998).
– Changes in sperm motility and kinematic parameters and changes in flagellar activity (Cancel et al. 2000; García-Herreros et al. 2005).
– Removal of AQN-1 from the sperm surface (Calvete et al. 1997; Töpfer-Petersen et al. 2008).
– Changes in permeability to extracellular calcium and increases in intracellular concentrations of this cation (Adeoya-Osiguwa and Fraser 2003).
– Phosphorylation of tyrosine residues of sperm head proteins (Petrunkina et al. 2004).
– A second reorganisation of membrane lipids and proteins, leading to sperm plasma and the outer acrosome membranes become fusogenic (Flesch and Gadella 2000; Tsai et al. 2007, 2010).

In short, the most significant changes during sperm capacitation entail the activation of several signalling pathways, such as sperm-specific adenylyl cyclase cAMP-dependent protein kinase A (PKA) and the reorganisation of proteins and lipids on the plasma membrane (Gadella et al. 2008). This change is facilitated by the removal of steroids (e.g. cholesterol) by acceptor proteins (such as BSA) and results in a more fluid membrane with an increased permeability to Ca^{2+}. This calcium influx produces, in turn, increased intracellular cAMP levels and, thus, an increase in motility (motility activation during fast and further hyperactivation during the slow capacitation events) (Baldi et al. 1996; Visconti et al. 1999a).

During the early stages of sperm capacitation, AQN-1, a seminal plasma protein that non-covalently attaches to the sperm membrane, is removed (Sanz et al. 1993; Dostàlovà et al. 1994; Calvete et al. 1997), thereby allowing AWN, AQN-3 and P47/SED1 to be accessible and able to interact with ZP-glycans (Flesch et al. 2001a).

Finally, when spermatozoa are fully capacitated, there is a destabilisation of the acrosomal sperm head membrane that allows greater binding ability of sperm to bind the zona pellucida of the oocyte (Tsai et al. 2007).

Some authors (Salicioni et al. 2007; Visconti 2009) have divided capacitation into two different types of events to facilitate consideration of the complex cascade of molecular events that occurs during this process (Fig. 7.1):

- Fast and early events, which comprise activation of sperm motility and start as soon as the sperm leave the epididymis, and
- Slow and late events that comprise changes in the pattern of movement (hyperactivation), the ability to carry out acrosome reaction stimulated by a physiological agonist and the phosphorylation of tyrosine in proteins (Baldi et al. 1996; Visconti et al. 1999a). These events take place within the oviduct.

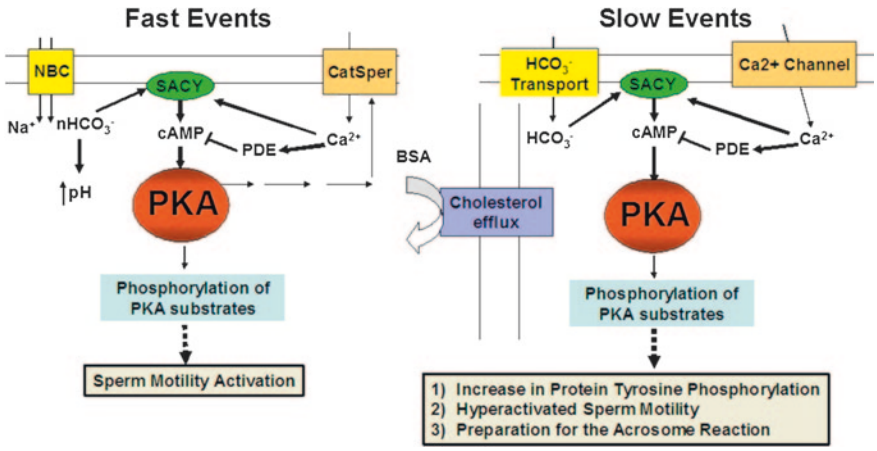

Fig. 7.1 Molecular basis of fast and slow events associated with sperm capacitation. In fast events (*left*), bicarbonate and Ca^{2+} stimulate sperm motility, through PKA and sACY. According to Visconti (2009), bicarbonate and Ca^{2+} are transported by a Na^{+}/HCO_3^{-} co-transporter (NBC) and a sperm-specific Ca^{2+} channel (CatSper). In slow events (*right*), spermatozoa acquire the ability to fertilise the egg. There are increases in tyrosine phosphorylation, sperm present hyperactivated motility and prepare to undergo the acrosome exocytosis (Visconti 2009; Reproduced with permission)

7.3 The Relevance of In Vitro Studies on Sperm Capacitation

According to Rodríguez-Martínez (2007), one inconvenience of investigating sperm capacitation is the difficulty in confirming in vitro experiments in in vivo conditions. In fact, it is quite difficult to study the intraluminal set of environments in the oviduct without disrupting the homoeostasis of this organ.

In recent years, however, many efforts have been made to study the events related to sperm capacitation. The set of molecular changes that occur during sperm capacitation as well as their regulation have been largely studied in in vitro conditions. This has provided relevant data about the pathways involved in this process, but with the inconvenience of lacking in vivo studies.

Thus, most of the studies developed for understanding which molecular mechanisms govern sperm capacitation have been performed using in vitro conditions. Before performing these in vitro experiments, seminal plasma proteins binding spermatozoa have to be eliminated, and this can be accomplished in the laboratory by density-gradient centrifugation of spermatozoa (Petrunkina et al. 2003; Yeste et al. 2009) or by washing and centrifugation of the sperm suspension (Fazeli et al. 1999). After washing, spermatozoa are in vitro capacitated by incubating in bicarbonate-enriched media (Holt and Harrison 2002; Puigmulé et al. 2011; Ramió-Lluch et al. 2012) (Fig. 7.2) which stimulates a series of downstream events (Gadella and Van Gestel 2004), including the stimulation of a special form of soluble adenylyl cyclase (sACY) (Litvin et al. 2003), which increases the intracellular

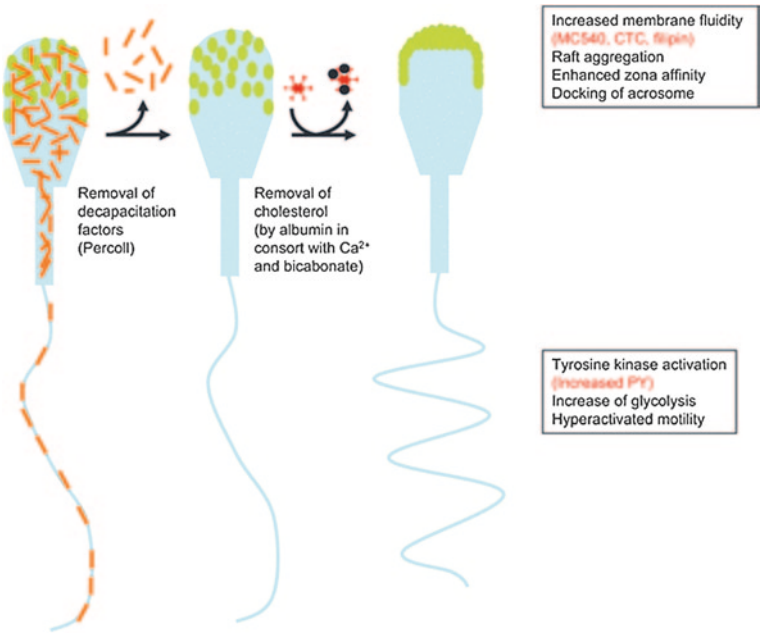

Fig. 7.2 Surface alterations during in vitro capacitation of boar spermatozoa. The decapacitation factors (in *orange*), originating from seminal plasma and adsorbed to the sperm surface during ejaculation, are removed by washing spermatozoa over a Percoll gradient. Subsequent incubation of spermatozoa in Tyrode's medium containing bicarbonate, fat-free albumin (in *red*) and Ca^{2+} cause the lateral redistribution, and partial removal, of cholesterol (in *black*). Consequently, lipid ordered domains (in *green*) aggregate into the apical ridge area of the sperm head. This allows the formation of a zona binding protein complex as well as the docking of the acrosome to the sperm head surface (Leahy and Gadella 2011; Reproduced with permission)

levels of cAMP and subsequently activates a signalling pathway dependent on PKA (Visconti et al. 1999a; Harrison 2004). Finally, in vitro capacitated spermatozoa are able to penetrate the cumulus layers and bind to ZP proteins.

Therefore, it is important to bear in mind that most of the data presented in this chapter about the molecular changes related to sperm capacitation have been obtained by conducting in vitro studies. This is undoubtedly a good approach to understand what happens in vivo, but can underestimate some important mechanisms of regulation. For this reason, further sections about the environmental conditions of the oviduct are expected to compensate this bias.

7.4 Role of the Oviduct in Sperm Capacitation

7.4.1 Lessons from In Vitro Studies

Direct contact of spermatozoa with oviductal epithelial cells (OEC), with oviductal explants, or with apical plasma membrane fractions, seem to delay rather than

promote sperm capacitation in porcine (Fazeli et al. 1999; Yeste et al. 2009), and other mammalian species, such as humans (Murray and Smith 1997), rabbits (Smith and Nothnick 1997) and sheep (Lloyd et al. 2009), thereby prolonging sperm function and survival (Rodríguez-Martínez et al. 2005). The viability-prolonging and capacitation-delaying effects of sperm are even observed when the male gametes are co-incubated with proteins isolated from apical plasma membranes (Satake et al. 2006), where HSPA8 plays a main role (Elliott et al. 2009; Lloyd et al. 2009, 2012). Indeed, although sperm motility is stimulated when uncapacitated boar spermatozoa are incubated with bicarbonate (Holt and Harrison 2002), the response to bicarbonate is reduced and the motility pattern of individual tracks is modified when these uncapacitated spermatozoa are co-incubated with apical plasma membranes (Satake et al. 2006). As will be further discussed, bicarbonate appears to be the key effector of sperm capacitation, and changes in the motility patterns are one of the features of this physiological process (Cancel et al. 2000). Thus, the non-response of spermatozoa to bicarbonate in terms of sperm motility clearly indicates that these apical plasma fractions delay sperm capacitation.

On the other hand, as widely reported in (Chap. 6), in in vitro co-culture, uncapacitated spermatozoa preferentially bind to OEC and then seem to capacitate quickly. In contrast, capacitation is not induced when uncapacitated sperm attach to non-reproductive cells (LLC-PK1) also in in vitro co-culture conditions (Fazeli et al. 1999; Yeste et al. 2009; see also Chap. 6).

All these data are not contradictory but need a careful explanation. In fact, it is worth noting that the storing function of sperm reservoir seems to be regulated by a concerted mechanism that is species-specific, since homologous co-culture is better than heterologous, and sperm-OEC binding is mediated by species-specific ligands (Pollard et al. 1991; Suarez et al. 1991; Suarez 2007).

7.4.2 Modulation of Sperm Capacitation Within the Oviduct

From the deposition site and upon their arrival at the ampullary–isthmic junction, where fertilisation takes place, spermatozoa are in contact with different environments within the female reproductive tract, spending a short time in the cervix and the uterus and staying longer in the oviduct (Rodríguez-Martínez 2007). In this context, the oviduct plays a relevant role in the modulation of sperm capacitation, in a process that occurs during sequential exposure of male gametes to these different female compartments (Yanagimachi 1994), and is actively and progressively coordinated in relation to ovulation (Hunter and Rodríguez-Martínez 2004; Rodríguez-Martínez 2007).

As discussed in the previous chapter, the oviduct provides a suitable environment for sperm transport, storage and capacitation, for oocyte collection, transport and maturation, for fertilisation and early embryo development; phenomena that occur during oestrus and metaoestrus (Rodríguez-Martínez 2007). Related to this, it is worth noting that the intraluminal fluid from the oviduct has been reported to

be involved in boar sperm capacitation (Rodríguez-Martínez et al. 2001; Tienthai et al. 2004), mediating the start of this process (Rodríguez-Martínez 2007).

As mentioned before, capacitation and molecular-related events have been hitherto investigated in in vitro conditions, with the inconvenience of having to confirm whether all these observations also take place in vivo (Rodríguez-Martínez 2007). One of the main problems that arises is trying to study intraluminal sets of environments without disrupting the homoeostasis of the organ. Thus, although the surface of ejaculated spermatozoa is known to be covered by spermadhesines and is accessible to lipid-binding components of uterine and oviductal fluids after mating and the sequence of capacitation-like changes has been largely addressed (Tardif et al. 2003; see Sects. 7.6 and 7.7), less is known about the modulation of sperm capacitation within the oviductal environment (Rodríguez-Martínez 2007).

Early studies showed that spermatozoa capacitate faster when they are exposed to the oviducts than when they are exposed to uterus and oviducts together (Hunter and Hall 1974). Under surgical conditions in pigs, spermatozoa are capacitated faster when they are first exposed to the caudal isthmus and then exposed to the cranial ampulla, than when they are only exposed to the latter (Hunter et al. 1998; Hunter and Rodríguez-Martínez 2004). In cattle, oviductal fluid from the isthmus has a greater ability to capacitate bull spermatozoa than the fluid collected from the ampulla (Killian 2004). These differences in time needed for capacitating male gametes could be related to the ability of the environment to clean the surface of the spermatozoa, so that the uterine and oviductal fluids modulate the velocity of sperm capacitation and allow the sequence of related events (Rodríguez-Martínez 2007).

During the pre-ovulation period, capacitation is not promoted by the sperm reservoir and spermatozoa remain attached (Hunter and Rodríguez-Martínez 2004; Rodríguez-Martínez et al. 2005). Around ovulation, spermatozoa are gradually released from the sperm reservoir (located at the isthmus) and go towards the venue of fertilisation, i.e. the ampullary–isthmic junction. However, it is worth to mention that the proportion of sperm leaving the isthmic sperm reservoir is always very small, probably only 5 % of them at most (Rodríguez-Martínez 2007). This finding suggests that capacitation events may occur at different times over several hours (see also Sect. 6.10).

Currently, the releasing mechanisms from sperm reservoir are not clear enough, as described in Chap. 6. Thus, on one hand, some authors have reported that hyaluronan (HA) could be involved in the detachment of spermatozoa from the sperm reservoir, by inhibiting the interaction between the spermadhesins covering the sperm surface and the oviductal cells (Liberda et al. 2006). On the other hand, it has also been hypothesised that release of spermatozoa from the sperm reservoir could be due to the capacitation-related changes themselves, which entail some proteins being removed. These changes would, in turn, involve a loss of binding sites in the sperm surface and thus diminish the affinity of spermatozoa to the epithelium (Fazeli et al. 1999). This would be supported by in vitro observations, since, as stated, spermatozoa bound to OEC capacitate sooner than spermatozoa bound to non-reproductive cells (LLC-PK1) (Yeste et al. 2009). Unfortunately, it is still not clear enough when capacitation starts and how spermatozoa are released from the reservoir.

To explain the modulating role of the oviduct on sperm capacitation, there is another interesting concept that we should bear in mind; that is, how sperm hyperactivation occurs in pigs. Sperm hyperactivation is defined as a late capacitation state in which spermatozoa exhibit vigorous and high amplitude flagellar beating. This phenomenon, which has been reported to occur in the oviduct of rodents and also during in vitro co-incubations of spermatozoa with oviductal explants in porcine and cattle, has been hypothesised as a putative mechanism by which mammalian spermatozoa detach from the reservoir (Suarez et al. 1992; Suarez 2007). However, no evidence has yet been reported that sperm hyperactivation also occurs in vivo in porcine (Rodríguez-Martínez 2007). One should note that in our description about molecular changes during late capacitation events (see Sect. 7.7), we repeatedly mention the term 'sperm hyperactivation'. This is due to the fact that the available knowledge about these changes mainly comes from in vitro studies, and care must thus be taken when trying to extrapolate this to what happens in vivo.

Considering all these aspects, Rodríguez-Martínez (2007) has proposed that the release of uncapacitated spermatozoa from the sperm reservoir is a constant and progressive process that occurs over a long time period, from pre- to post-ovulation, rather than massively at the moment of ovulation. This hypothesis is supported by other findings demonstrating that uncapacitated spermatozoa recovered from the seminal reservoir of inseminated sows can be in vitro capacitated by incubation in a medium containing bicarbonate, a capacitation effector, at concentrations similar to those recorded in vivo in the ampullary–isthmic junction and in the ampulla during the peri-ovulatory period (Tienthai et al. 2004).

On the other hand, the modulating function of the oviduct on sperm capacitation in vivo seems to be related to regional differences throughout the oviduct, apart from reported differences in the ovarian state. Indeed, as stated in Chap. 6, the oviduct can be anatomically divided into different parts. The first part of the oviduct forms the sperm reservoir, which involves the adhesion of spermatozoa to OEC and provides a safe environment for spermatozoa. Here, a portion of the inseminated spermatozoa undergoes a period of storage within a restricted tubal segment. The period of this storage time, when sperm cells are in a quiescent state within a restricted segment of the female oviduct, lasts from hours to days (Rodríguez-Martínez 2007).

The second part, the ampulla, would be the place where capacitation takes place. The suggestion of a regional difference in sperm capacitation through segments is supported by in vivo observations, since boar spermatozoa having capacitation-like motility (increase of VCL, decrease of LIN, etc.) are only retrieved from the ampulla (Suarez et al. 1992). In addition, the influence of the oviductal region on sperm capacitation is also supported by other studies conducted with oviductal fluid, since the peri-ovulatory fluid collected at the ampullary–isthmic junction increases the number of ejaculated spermatozoa showing hyperactivation-like motility (Nichol et al. 1997).

Finally, Fraser (2010) has recently suggested that oviductal fluid contains specific regulatory ligands, like adenosine, calcitonin and fertilising promoting peptide (FPP), which can accelerate sperm capacitation after release from the oviductal reservoir. This aspect will be extensively discussed in Sect. 7.11.

7.5 Effectors of Sperm Capacitation

7.5.1 Introduction: Relevant Molecules for Sperm Capacitation

The literature has provided extensive evidence that bicarbonate, calcium, and serum albumin (BSA) are molecules that are present in the female reproductive tract and play a critical role during sperm capacitation (Yanagimachi 1994; de Lamirande et al. 1997; Fraser 2010). These three molecules promote cytoplasmic and plasma membrane changes in spermatozoa, which must occur before cells can complete capacitation, undergo acrosome reaction, and fertilise the oocyte.

The mechanisms by which these molecules are able to promote capacitation at the molecular level, by modulating adenylyl-cyclases and cAMP levels, plasma membrane architecture, and protein phosphorylation (Fraser 2010), have been an active area of research in recent years (Salicioni et al. 2007).

7.5.2 The Crucial Role of Bicarbonate in In Vivo Capacitation

As explained above, most of the capacitation-related events have been observed in in vitro experiments, by using media containing higher concentrations of bicarbonate (Holt and Harrison 2002; Gadella and Van Gestel 2004). Although bicarbonate appears to be a key effector in these in vitro experiments, it is not known exactly whether it is the sole effector.

For the time being, bicarbonate has been considered to be the effector, or at least one of the key effectors, of sperm capacitation in porcine, bovine and equine species (Harrison and Gadella 2005; Tienthai et al. 2004; Rodríguez-Martínez 2007) within the oviductal environment. This is consistent with the variations of bicarbonate levels along the oviductal segments towards the site of fertilisation, and with the relationship between boar sperm motility and bicarbonate concentrations. In epididymal cauda, for example, spermatozoa are in a quiescent state because of lower levels of bicarbonate and pH when compared to ejaculated spermatozoa (Rodríguez-Martínez et al. 1990). In the case of the oviduct, bicarbonate levels are lower in the caudal isthmus than in the ampulla–isthmic and ampulla regions (Rodríguez-Martínez 2007), and also vary during the oestrous cycle. In this regard, this concentration could be optimal around ovulation, since, as mentioned, oviductal fluid collected during the peri-ovulatory stage elicits sperm capacitation (Rodríguez-Martínez et al. 2001).

Therefore, all these data support the role of bicarbonate in triggering the capacitation of boar spermatozoa in vivo (Tienthai et al. 2004). Indeed, according to this hypothesis, sperm capacitation would not be induced in the sperm reservoir because of the low levels of bicarbonate that are non-capacitating, while capacitation would occur at the ampullary–isthmic junction where the levels of

bicarbonate are higher. Thus, the progression of a spermatozoon through the oviduct is enough to elicit its capacitation, when it does not take place in the sperm reservoir, and when the concentration of bicarbonate in the oviductal fluid is adequate (Rodríguez-Martínez et al. 2005).

This hypothesis also suggests that, at least in vivo, the release from sperm reservoir is gradual and might occur prior to eliciting sperm capacitation-related events (Rodríguez-Martínez 2007). Therefore, if we assume that bicarbonate is the key effector of sperm capacitation and that the sole progression of spermatozoa through the oviduct leads to their capacitation, and if we bear in mind that spermatozoa die when they do not encounter any oocyte, it is likely that the gradual release of spermatozoa from the sperm reservoir acts as a mechanism to warrant the availability of capacitated spermatozoa for the long interval lasting from sperm deposition to ovulation (Rodríguez-Martínez et al. 2005).

Although the molecular changes during sperm capacitation will be taken up again in further specific sections (Sects. 7.6 and 7.7), we briefly describe now, to complement this section, the changes produced on sperm by the presence of bicarbonate. As such, bicarbonate induces the remodelling of the sperm plasma membrane by firstly increasing its fluidity (Harrison and Miller 2000; Rodríguez-Martínez 2007; Puigmulé et al. 2011), despite individual spermatozoon differences existing in terms of phospholipid scrambling (Gadella and Van Gestel 2004). After lipid scrambling and due to the presence of both bicarbonate and BSA, there is an efflux of cholesterol molecules, which are redistributed or removed from the sperm head domain (Flesch et al. 2001b; Gadella and Van Gestel 2004). This event consists of the activation of tyrosine kinases, and the subsequent tyrosine phosphorylation of sperm proteins (Ficarro et al. 2003).

Bicarbonate is also involved in the migration of seminolipid, a sperm-specific glycolipid, from the apical head to the equatorial domain (Gadella et al. 1995) (Fig. 7.3). When bicarbonate levels are low, seminolipid molecules stabilise the plasma membrane, thereby impeding the acrosome reaction in the apical head domain of uncapacitated spermatozoa. In contrast, when bicarbonate levels are higher, seminolipids are translocated to the equatorial region, which diminishes their protective role. The outer membrane is then destabilised and becomes fusogenic to undergo acrosome reaction after binding with ZP-proteins (Rodríguez-Martínez 2007; Tsai et al. 2007). In contrast to the apical head sperm region, the post-acrosomal domain that fuses with the oolemma when fertilisation occurs does not react to higher levels of bicarbonate and keeps its stability (Gadella et al. 1995; Rodríguez-Martínez 2007).

High levels of bicarbonate also stimulate a sACY, the unique sACY-type present in spermatozoa (Signorelli et al. 2012), which triggers the production of cAMP and activates, in turn, the PKA (Chen et al. 2000; Harrison 2004; Botto et al. 2010). The activation of this cAMP-dependent pathway activates phospholipid scrambling in the apical sperm membrane, increases lipid disorder and membrane fluidity, as merocyanine-540 staining can show (see Sect. 9.3.6.2), and facilitates cholesterol redistribution over the sperm head (Gadella and Harrison 2002), in one of the key early/fast events of sperm capacitation. After their redistribution, cholesterol molecules are extracted and are then taken by acceptor proteins (Gadella 2008a).

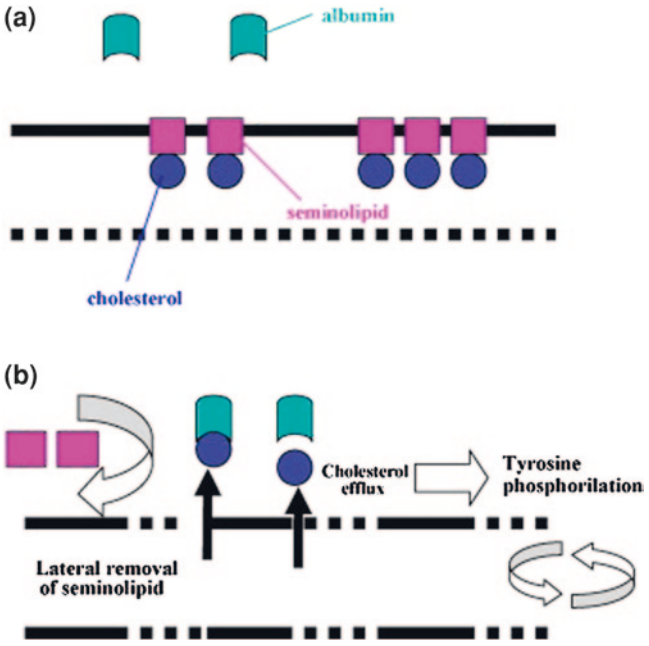

Fig. 7.3 Cholesterol efflux from the sperm plasma membrane during sperm capacitation. In ejaculated spermatozoa (**a**), the distribution of phospholipids is asymmetric and cholesterol levels are relatively high. The phospholipids scramblase is not activated and the cholesterol cannot be depleted by albumin. The sperm-specific glycolipid seminolipid is concentrated in the apical plasma membrane. In sperm capacitated in the presence of albumin, bicarbonate and Ca^{2+}, scramblase is activated via a bicarbonate adenylate cyclise-PKA signalling pathway. As a result, the phospholipids asymmetry of the plasma membrane collapses and this permits an albumin-mediated efflux of cholesterol and a lateral membrane removal of seminolipid (Witte and Schäfer-Somi 2007; Reproduced with permission)

In short, all these molecular changes modify the physicochemical properties of sperm membranes, thereby allowing further late changes of sperm capacitation, such as the fusion of plasma membrane and outer acrosome membrane (Gadella 2008a; Gadella et al. 2008). Therefore, the role of bicarbonate is crucial in sperm capacitation-related events since it primes the spermatozoon to interact with ZP, render the apical sperm head membrane fusogenic and undergo acrosome reaction/exocytosis (Gadella and Van Gestel 2004).

7.5.3 The Role of Other Oviductal Fluid Components (HA and BSA) on Sperm Capacitation

As already mentioned in this and Sect. 6.5.3, oviductal fluid also influences sperm capacitation. In bovine species, for example, the oviductal fluid collected during standing oestrus increases the percentage of capacitated spermatozoa (Bergqvist

et al. 2006). In porcine species, preovulatory fluid collected from the isthmus seems to maintain sperm viability without inducing sperm capacitation, while post-ovulatory fluid from the same region increases the percentage of capacitated spermatozoa, thereby indicating a different composition of oviductal fluid depending on the stage of the oestrus cycle. In fact, only the oviductal fluid collected at the peri-ovulatory period induces sperm capacitation (Rodríguez-Martínez 2007).

Oviductal fluid, as explained in Sect. 6.5.3, contains sulphated and non-sulfated glycosaminoglycans. Within sulphated glycosaminoglycans, dermatan sulphate induces capacitation, while the effects of HA appear to be in conflict with the literature because it delays capacitation after 3 days of storage at 15 °C (Yeste et al. 2008) and in vivo (Rodríguez-Martínez et al. 2001), but also seems to induce it in vitro conditions. Indeed, HA causes a slight capacitation in vitro, without triggering the acrosome reaction in bovine and boar semen after incubating at 38 °C (Bergqvist et al. 2006). In contrast, when boar spermatozoa are flushed from the sperm reservoir during the pre-ovulatory period (in vivo conditions) and they are further co-incubated with HA and bicarbonate, HA appears to prevent the induction of sperm capacitation (Tienthai et al. 2004). Similarly, when ejaculated spermatozoa are extended with a short-term extender and preserved at 15 °C, HA also delays sperm capacitation after 3 days of storage (Yeste et al. 2008).

Amidst this controversy, heparin and dermatan sulphate, both glycosaminoglycans like HA, have been regarded as sperm capacitation inductors in bull spermatozoa, but they increase the number of dead spermatozoa without increasing the percentage of capacitated spermatozoa in frozen-thawed spermatozoa (Bergqvist et al. 2007).

Finally, another component of oviductal fluid that is involved in sperm capacitation is BSA. This molecule is believed to function as a sink for the depletion of sperm plasma membrane cholesterol and can be replaced by other cholesterol-binding compounds such as cyclodextrins to induce capacitation (Salicioni et al. 2007).

7.6 Early/Fast Capacitation Events: Bicarbonate, Calcium and Activation of Sperm Motility

7.6.1 Introduction

As mentioned, controversy exists as to whether early/fast capacitation events have to be considered as part of the capacitation process or not. These rapid events occur a few seconds after ejaculation, and therefore not within the oviduct, and they are related to high concentrations of calcium and bicarbonate present in seminal fluid (Visconti 2009). Since capacitation requires several hours, and we are now referring to early/fast events, it is quite reasonable to consider that these changes are required to initiate capacitation prior to other events that continue and complete capacitation. For this reason, Fraser (2010) has rightly considered that these early/fast changes of sperm capacitation form the basis for eventual acquisition of fusibility characteristics required for acrosome reaction.

It is very important to emphasise that fast changes occur following release of spermatozoa into a bicarbonate-containing medium, also in in vitro conditions (Visconti et al. 1999a; Gadella and Harrison 2000; Holt and Harrison 2002; Harrison 2004). In this regard, we should also bear in mind what has previously been discussed about the main role of bicarbonate as a sperm capacitation effector (see also Sect. 7.5.2).

Bicarbonate enters the sperm through the co-transporter Na^+/bicarbonate (Demarco et al. 2003), producing an increase in intracellular pH and activating the sACY, which, as mentioned before, is the only type of sACY present in spermatozoa (Signorelli et al. 2012). Evidence has repeatedly indicated that this atypical cyclase is the main target of bicarbonate during sperm capacitation (Fraser 2010). In contrast to transmembrane adenylyl-cyclases, this soluble form is activated by bicarbonate and Ca^{2+} but does not respond to activators of Gs, the G protein stimulator of transmembrane cyclases, such as cholera toxin and non-hydrolyzable analogues of GTP (Visconti 2009).

During these fast/early capacitation events, there are two bicarbonate-mediated changes that occur almost simultaneously. One involves changes in the sperm plasmalemma (membrane fluidisation), while the other is known as sperm motility activation (Fraser 2010), and the latter event occurs faster than the former (Harrison 2004; Harrison and Gadella 2005). However, and despite both events taking place together to some extent, they will be described separately in the following two subsections.

7.6.2 Plasma Membrane Changes During Early Events

Bicarbonate activates a phospholipid scramblase, an enzyme that translocates membrane phospholipids between the outer and inner leaflets of the membrane and causes rapid changes in the phospholipid asymmetry of this membrane (Gadella and Harrison 2000; Harrison and Gadella 2005). Indeed, this scramblase-activation results in translocation of phosphatidylserine and phosphatidylethanolamine to the outer leaflet, rather than being mostly or exclusively located in the inner leaflet. It is worth noting that although translocation of these lipids in the outer leaflet mainly occurs in the acrosomal region, sACY has been identified in the midpiece of the tail (Hess et al. 2005). All these changes reduce membrane stability (Gadella and Harrison 2002), so that, in turn, this scramblase-initiated destabilisation makes cholesterol available to external receptors. Cholesterol can then be more easily removed, and this leads to a rapid collapse of sperm plasmalemma asymmetry (Salicioni et al. 2007; Visconti 2009) (Fig. 7.3). The cholesterol removal by acceptor proteins, such as BSA, is considered a late capacitation event (Gadella and Harrison 2002) and will be considered in greater detail in Sect. 7.7.

Botto et al. (2010) have studied the biochemical events involving membrane architecture in spermatozoa exposed to bicarbonate before the extraction of cholesterol molecules. These authors have paid special attention to the relocation of two

endocannabinoid system receptors (see also Sect. 6.9); cannabinoid receptor type 1 (CBR1) and transient receptor potential cation channel 1 (TRPV1) during early/fast capacitation events, since both proteins are known to be involved in capacitation-related spermatozoa signalling. Interestingly, these researchers have observed that bicarbonate promotes massive membrane remodelling in the absence of extracellular proteins. This membrane re-organisation entails a series of changes.

Firstly, when spermatozoa are in vitro incubated with bicarbonate, there is an increase in protein content in lipid microdomains, also known as detergent-resistant membrane-domains (DRMDs), since they are insoluble in non-ionic detergents and have light buoyant/floating density after centrifugation in a discontinuous sucrose gradient (Botto et al. 2008; Pike 2009). These lipid microdomains, which are rich in cholesterol and sphingolipids, compartmentalise the sperm membrane. Previous reports in porcine (Shadan et al. 2004; Van Gestel et al. 2005) and other mammalian species (Travis et al. 2001) have suggested that such microdomains are involved in the acquisition of fertilising capacity. In a study that we are conducting at present, it would appear that sperm DRMDs play a relevant role not only in capacitation but also in the changes in sperm function after boar sperm cryopreservation (Yeste et al. 2012, unpublished).

To date, caveolae and lipid rafts are the two proposed types of lipid microdomains (Botto et al. 2010). Anderson (1998) has reviewed the concept and function of caveolae, which are lipid raft-enriched domains present on the plasma membrane of many eukaryotic cell types (Anderson 1998). Caveolae can present different shapes, including flat, vesicular and tubular and they can be either open at the cell surface or closed off forming an exocytic/endocytic compartment. Although all cells have plasma membrane domains with the biochemical features of caveolae, only a subset of these membranes display the flask-shaped morphology typical of caveolae (Anderson 1998). They play a role in cell signalling, cholesterol homoeostasis, clathrin-independent endocytosis, transcytosis and potocytosis (Matveev et al. 2001; Parton and Richards 2003). With regard to their biochemical composition, caveolae contain lipids, such as glycosphingolipids and cholesterol, and proteins, such as caveolin, which is the main integral protein of these domains, presenting their carboxyl and amino termini located in the cytosol and a hydrophobic loop inserted into the membrane (Glenney and Soppet 1992; Rothberg et al. 1992). These caveolins bind to cholesterol (Murata et al. 1996) and oligomerise, thereby stabilising the membrane domain and defining the size and the shape of caveolae (Fernández et al. 2002; Parton and Simons 2007). Within caveolins, caveolin-1 and caveolin-2 are ubiquitously expressed in mammalian cells. As far as lipid rafts are concerned, it is important to note that they are flat domains containing high amounts of glycosphingolipids and glycosylphosphatidylinositol-anchored proteins (Brown 2006). The formation of these lipid rafts depends on lipid–lipid interaction (Lindner and Naim 2009).

The increase in protein content in DRMDs is associated with a significant increase in caveolae and CD55, but does not alter the percentages of cholesterol in these DRMDs, thereby suggesting that this reorganisation of the sperm membrane architecture is independent from cholesterol extraction. In addition, this finding

suggests, for the first time, that the reorganisation of DRMDs, which is one of the features of sperm capacitation, starts before cholesterol extraction. These results have led the sequence of capacitation events to be reconsidered, since until the report by Botto et al. (2010), membrane remodelling was believed to take place only when extracellular proteins or cholesterol-extracting molecules were present (Choi and Toyoda 1998).

Bicarbonate has also appeared to promote the migration of the two mentioned receptors of endocannabinoids (CBR1 and TRPV1). Thus, although the CBR1 content is low in DRMDs before membrane remodelling, the amount of this protein in these lipid rafts increases after bicarbonate exposure. In DRMDs extracts, CBR1 appears in a double band: one corresponds to the unglycosylated form (54 kDa), and the other corresponds to the glycosylated (active) form (65 kDa) (Botto et al. 2010). According to Maccarrone et al. (2005), CBR1 would slow the tendency of plasma membrane to become unstable, thereby avoiding the premature loss of acrosome integrity. The signal transduction of the activated receptor is a cAMP-dependent pathway.

In the case of TRPV1, its amount is low in DRMDs before membrane reorganisation, but it also increases after bicarbonate exposure (Botto et al. 2010). This receptor, modulated by ionic intracellular concentrations, is involved in the regulation of sperm transmembrane potential, in the intracellular calcium levels and in actin polymerisation (Bernabò et al. 2010a), all of them being capacitation-related events.

All these results lead these authors to hypothesise some role of these endocannabinoid receptors in the control of sperm–oocyte interaction. In addition, the role of these two endocannabinoid receptors in the acquisition of sperm fertilising ability does not depend on their action mechanism, but it relies on the membrane remodelling process itself, which affects the biochemical localisation of these molecules.

On the other hand, Botto et al. (2010), based on research by Asano et al. (2009) in mice and Cummerson et al. (2006) in humans, have proposed that during their formation, lipid rafts are also present in the acrosome membrane. These authors also hypothesised that, after bicarbonate exposure, some DRMDs component could originate from the outer acrosomal membrane and be later transferred to the sperm surface via an exocytotic process (Botto et al. 2010). This hypothesis seems to be reasonable given that plasma membrane and outer acrosomal membrane fuse with each other at the moment of acrosome reaction.

In short, Botto et al. (2010) have suggested that sperm capacitation entails a quite complex and integrated functional dialogue between the endocannabinoid system and the microdomain architecture of plasma and outer acrosomal membranes. All these events take place prior to the fusion of plasma and outer acrosome membranes, which will be further described in Sect. 7.7 about late/slow capacitation events.

Finally, we must mention that controversy exists as to what extent cholesterol removal is the signal that leads to membrane destabilisation. Thus, even though some studies have reported that loss of cholesterol is the initial event leading to

membrane destabilisation, to cAMP-increases and to the activation of PKA (Visconti et al. 1999b, c, 2002), other evidence suggests that the collapse of membrane asymmetry precedes and facilitates subsequent cholesterol removal (Flesch et al. 2001b). As stated, the other changes in the sperm membrane that occur during late/slow events will be reconsidered in a further specific section (Sect. 7.7).

7.6.3 Removal of AQN-1 from Sperm Surface During Early Capacitation Events

Another relevant change that is produced during an early stage of in vitro capacitation is the release of AQN-1 from the sperm surface (Ekhlasi-Hundrieser et al. 2005). This spermadhesin, which plays a relevant role in the formation of pig sperm reservoir as described in Sect. 6.7.3.3, masks other sperm surface associated proteins (AQN-3, AWN and P47/SED1) that are involved in the interaction of the spermatozoa with the oocyte's zona pellucida (Figs. 6.4 and 8.2).

At a very early stage of sperm capacitation, sperm membrane is destabilised and AQN-1 is removed from the sperm surface. This leads to the release of the spermatozoa from the oviductal reservoir and allows AWN, AQN-3 and P47/SED1 to become accessible and thus make their interaction with ZP possible (Töpfer-Petersen et al. 2008). Interaction of these three sperm surface associated proteins to ZP will be taken up again in Fig. 8.2.

7.6.4 Activation of Sperm Motility

Activated sACY increase cAMP levels, and the increased levels of this second messenger stimulate PKA. Once activated, PKA phosphorylates various target proteins that are presumed to initiate several signalling pathways. Notwithstanding, in sperm exposed to bicarbonate, cAMP-increases to a maximum within 60 s, and the rise in PKA-dependent phosphorylation begins within 90 s (Salicioni et al. 2007). In contrast, increases in protein tyrosine phosphorylation only occur later (Harrison 2004).

One of the PKA-activated pathways modulates the response of sperm-specific calcium voltage-gated channels (CatSper), which produces changes in the membrane potential and increases the intracellular concentration of Ca^{2+} (Wennemuth et al. 2003; Signorelli et al. 2012). All these molecular changes participate in the activation of sperm motility (Fig. 7.1). This can be explained by the role of bicarbonate and Ca^{2+} on sperm motility before and after ejaculation. Indeed, although sperm stored in the cauda epididymidis consume oxygen at a high rate, they are immotile. Conversely, when spermatozoa are released from the epididymis and they come into contact with high bicarbonate and Ca^{2+} present in the seminal fluid, there is an immediate activation of the flagellum movement.

7.7 Late/Slow Capacitation Events: Hyperactivation, Membrane Remodelling and Protein Phosphorylation

7.7.1 Introduction

In contrast to the fast activation of sperm motility, other capacitation-associated processes require longer incubation periods. These slower processes can be accomplished in vitro by using sperm incubated in defined media. In all cases, in vitro capacitation media must contain a protein source that is usually BSA; and an assortment of ions including bicarbonate and Ca^{2+} (Salicioni et al. 2007; Visconti 2009). The relevance of in vitro studies in sperm capacitation has been briefly mentioned in Sect. 7.3.

It is considered that late/slow sperm capacitation events start after cholesterol efflux from sperm plasma membrane mediated by BSA, and take place in the oviduct. This cholesterol removal increases membrane lipid disorder and fluidity, allows reorganisation of lipid-raft membrane domains and permits spermatozoa to maintain high levels of bicarbonate (Cross 2004). In addition during this phase, PKA phosphorylates serine and threonine residues of several proteins. Such Ser- and Thr phosphorylation results, in turn, in an increase in the phosphorylation of tyrosine residues, through the activation of protein kinases or the inhibition of protein phosphatases (Signorelli et al. 2012). Finally, this signal transduction pathway leads to a completion of sperm capacitation, which is manifested by the following events (Baldi et al. 1996; Salicioni et al. 2007):

1. Hyperactivation
2. Chemotactic behaviour
3. The ability of spermatozoon to trigger acrosome reaction induced by ZP or progesterone
4. The ability of spermatozoon to fertilise an oocyte.

7.7.2 Calcium and Hyperactivated Motility

Yanagimachi (1970), working with hamster sperm, was the first to report the existence of hyperactivated motility in mammalian spermatozoa. Hyperactivated motility consists of very active thrusting and asymmetric flagellar movements. In vivo, these changes in the specific motility/kinetic patterns occur in the oviduct. In addition, these changes can also be observed after proper stimulation in vitro, so that these findings have allowed more about this phenomenon to be learnt. We should bear in mind, as previously mentioned, that hyperactivation, without the so vigorous flagellar movement reported in mice, has been observed in vitro but not yet in in vivo conditions in boar spermatozoa (Rodríguez-Martínez 2007; see also Sect. 7.4.2). Moreover, under in vitro conditions, the percentage of hamster spermatozoa exhibiting hyperactivated motility (about 40 %) is quite higher than that of boar

spermatozoa, where only few sperm show this motility pattern. Therefore, and although the main data about sperm hyperactivation mentioned in this subsection come from rodents rather than from pigs as studies about the latter are scarce, it is worth remembering that this knowledge can not be directly extrapolated to pigs but care must be taken when trying to explain why happens in pigs from mice studies.

Hyperactivation is a calcium-dependent process and is required for successful fertilisation of zona-intact oocytes (Suarez 2008). Apart from motility activation in early-capacitation events, CatSper proteins (one, two, three and four), which we remember are sperm-specific calcium channels, have also been reported to be involved in motility hyperactivation (Kirichok et al. 2006; Qi et al. 2007; Fraser 2010). The main role of these calcium channels in hyperactivated motility is supported by previous studies made in male mice null for any one of the four CatSper proteins. These males are infertile and are not able to penetrate zona-intact oocytes (Ren et al. 2001; Quill et al. 2003; Jin et al. 2007; Qi et al. 2007). In addition, Ho et al. (2009), also working in mice, demonstrated that CatSper-null spermatozoa were unable to show hyperactivated motility and were unable to detach from the oviductal epithelium and thus reach the site of fertilisation.

7.7.3 Reorganisation of Sperm Membrane During Late/Slow Capacitation Events

As is widely known, one of the most relevant features of sperm capacitation is related to the changes and reorganisation of membrane lipids and proteins that affect all the subcellular compartments. This event concomitantly takes place with the activation of several signalling pathways such as sperm sACY, cAMP-dependent PKA and protein tyrosine phosphorylation (Flesch and Gadella 2000; Gadella et al. 2008). In a more accurate analysis, remodelling of sperm membranes during capacitation has been proposed as a two-step model (Flesch et al. 2001b):

- First, changes in sperm plasma membrane that involves a quick exposure of phosphatidylserine, detectable after 5 min and completed within 10 min; and a later exposure of phosphatidylethanolamine, which is completed after 30 min (Gadella and Harrison 2002). This would correspond to what occurs during the early/fast capacitation events (see Sect. 7.6.2).
- Second, and after between 2 and 4 h, cholesterol molecules are extracted with the involvement of acceptor proteins, and sperm plasma and outer acrosomal membranes become stable docked (Gadella 2008a; Gadella et al. 2008). This completes membrane remodelling and allows spermatozoa to acquire fertilising ability. These changes would correspond to what occurs during late/slow capacitation events and will be treated in more detail in the present section.

Both during early/fast and late/slow capacitation events, membrane remodelling entails the participation of specific DRMDs, as Gadella (2008a) and Botto et al. (2010) have proposed. The participation of DRMDs in early/fast capacitation

events has been previously discussed in Sect. 7.6.2. In the case of late/slow events, the completion of capacitation induces a close apposition/interaction of the apical plasma with the outer acrosome membrane. Indeed, when sperm membranes of capacitated and uncapacitated spermatozoa are isolated using nitrogen cavitation and differential centrifugation steps, it appears that membrane fractions of capacitated spermatozoa are bilamellar, while those of uncapacitated spermatozoa are unilamellar (Tsai et al. 2007, 2010). Furthermore, the outer acrosomal membranes isolated from capacitated spermatozoa present a significantly higher number of PNA-binding sites than those isolated from uncapacitated spermatozoa (Flesch et al. 1998).

In the formation of these bilamellar structures after capacitation as well as during acrosome exocytosis (see also Chap. 8), SNARE complexes play a key role. The acronym 'SNARE' stands for proteins from the Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor. These proteins are involved in calcium-mediated membrane fusion processes and in exocytosis-mediation in several cells types, such as neutrophils (Chen and Scheller 2001; Jahn and Scheller 2006; Verhage and Toonen 2007). Proteins belonging to the SNARE family have also been suggested to play a relevant role in mammalian fertilisation (De Blas et al. 2005; Ramalho-Santos et al. 2000; Tomes et al. 2002).

Both bilamellar membrane structures and trans-SNARE complexes formed after sperm capacitation remain stable after their post-cavitation isolation (Tsai et al. 2007). These trans-SNARE complexes appear to be highly stable, resistant to high temperature and to reducing conditions, and only fall apart into SNARE monomers when a treatment combining reducing agents and high temperature is provided. Related to this, Tsai et al. (2010) have suggested that SNARE-monomers gain extra protection when trans-SNARE complexes form large functional DRMDs to withstand the changes (i.e. temperature, pH etc.) in the surrounding environment.

The high stability of SNARE complexes can be explained because SNARE monomers are assembled prior to membrane fusion (Bennet and Scheller 1994; Jahn and Sudhof 1994). This assures the formation of stable SNARE complexes allowing successful acrosome exocytosis upon calcium stimulation. This idea is in agreement with Ackermann et al. (2008) and Heydecke et al. (2006), who suggested that a DRMD-associated scaffold protein governs the acrosome reaction in mammalian spermatozoa, and this may serve to direct the acrosome and plasma membrane SNAREs into the right membrane topology for the formation of stable ternary complexes (Tsai et al. 2010).

SNARE interactions have been related to the calcium-dependent acrosome reaction in mouse spermatozoa (Heydecke et al. 2006), while in boars Tsai et al. (2007) have observed that SNARE proteins cluster into DRMDs upon capacitation, forming a ternary trans-SNARE complex. Indeed, after capacitation but not before, there is a formation of an 80 kDa SNARE-complex, made up of SNAP23 (a specific protein of outer acrosome membranes) and VAMP3 (a specific protein of plasma membrane). Acrosomal SNAP23 is present in the outer acrosome membrane and in the plasma membrane of capacitated spermatozoa, while VAMP3, which has a molecular mass of 16 kDa, is specific for the plasma membrane and

is only present in monomeric form in uncapacitated spermatozoa. In contrast, it appears in the 80 kDa band in capacitated spermatozoa (Tsai et al. 2007, 2010). As a result of SNAP23/VAMP3 interaction, the acrosome membrane becomes stable docked to the apical sperm head plasma membrane, without the occurrence of fusions between the two interacting membranes (Fig. 7.4).

Both uncapacitated and capacitated spermatozoa present SNARE complexes containing VAMP-1 and -2 and syntaxins 2 and 2. This suggests that syntaxin 2/3 and VAMP 1/2 could be interacting SNARE proteins and form an additional pool of SNARE complexes (Tsai et al. 2010). These molecules do not seem to participate in membrane docking, but their exact role in the regulation of the acrosome exocytosis still remains unknown. Syntaxins 1B, 2 and 3 are predominantly present in sperm plasma membrane in uncapacitated spermatozoa, and syntaxin 2 and 3 are present in their monomeric form of 36 kDa (Tsai et al. 2010). However, the contribution of these two syntaxins after capacitation to the 80 kDa protein complex is marginal, so that it does not seem to be the primary cognate Q-SNARE with SNAP 23 and VAMP 3. In contrast, syntaxin 1B is present in the 80 kDa SNARE complex formed after capacitation, so that Tsai et al. (2010) have suggested that syntaxin 1B/SNAP 23/VAMP 3 are the interacting SNARE proteins in the 80 kDa trans-SNARE complex (Fig. 7.4).

From the previously mentioned SNARE proteins, it is worth noting that SNAP 23 shows a three-time higher DRMD-association affinity than SNAP 25 (Salaun et al. 2005; Tsai et al. 2010).

As mentioned, the stable docking of the outer acrosome membrane to the plasma membrane after sperm become completely capacitated is not accompanied by membrane fusions (Tsai et al. 2010). This entails the bilamellar structure remaining intact after docking and priming and the two interacting membranes persisting together as bilamellar membrane structure. Thus, formation of ternary trans-SNARE complexes is not sufficient to induce the AR but require additional stimuli or the removal of inhibitory components to allow fusion reaction. Therefore, binding of spermatozoa to intact-ZP is needed to increase calcium levels, via the opening of a transient receptor potential (TRP) channel (Florman et al. 2008). In this regard, it is worth noting that the area where the docking and priming of the plasmalemma and the acrosome occurs is the same area where the spermatozoon binds to the zona pellucida (Flesch and Gadella 2000). In addition, this area corresponds to the area where sperm membrane DRMDs have been shown to aggregate during capacitation treatments (Tsai et al. 2007; Van Gestel et al. 2005, 2007). According to Tsai et al. (2010), the stability and extended area of these docked and primed membranes might explain why acrosome membrane fusions after sperm bind to ZP. These interactions and fusions between the two membranes involved in the acrosome reaction (i.e. acrosome and plasma membrane), and which result in a generation of mixed vesicles, are thus separated processes in mammalian fertilisation. In addition, the formation and stabilisation of SNARE complex during docking and priming between plasma and acrosome membrane has also been suggested as a mechanism for preventing acrosome exocytosis in the absence of ZP-induced calcium influx (Tsai et al. 2010).

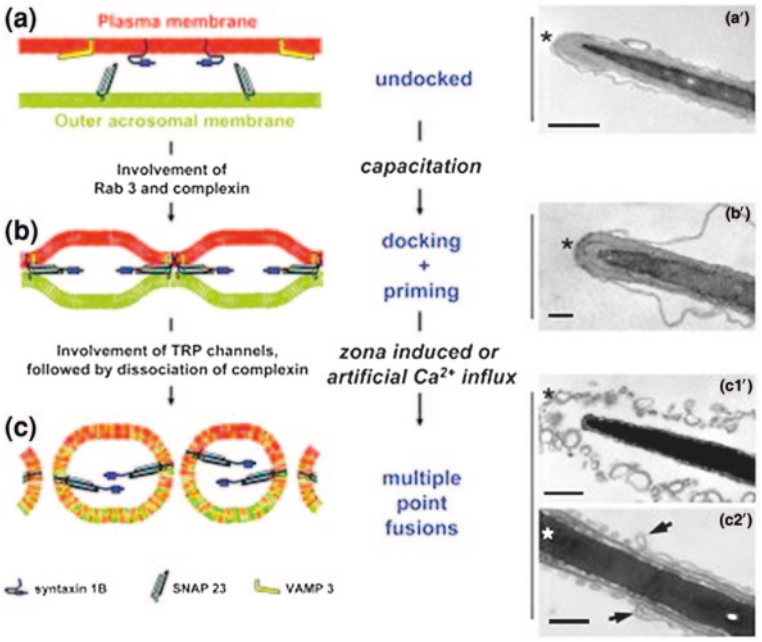

Fig. 7.4 Model for capacitation-induced stable docking of the acrosome to the sperm plasma membrane proposed by Tsai et al. (2010). In uncapacitated spermatozoa (**a**), plasma membrane (in *red*) and outer acrosomal membrane (in *green*) are not associated together. Syntaxin 1B (*purple blue*) and VAMP 3 (*yellow*) are located at the plasma membrane and SNAP 23 (*light blue*) is found at the outer acrosome membrane. The undocked plasma membrane appears as loose arrangement at the entire head area due to the osmotic effect by EM processing (**a′**). In capacitated spermatozoa (**b**), outer acrosomal membrane is stably docked to the apical plasma membrane and a stable ternary-SNARE complex is formed without membrane fusion (**b′**). Finally, when spermatozoa bind to the ZP (**c**) there is the fusion between membranes. However, although mixed vesicles of the apical plasma and outer acrosomal membranes are the result of the multipoint fusions characteristic for sperm acrosome exocytosis (**c1′**), there is no fusion at the equatorial sperm head area (distal from the arrows indicated in **c2′**) (Tsai et al. 2010; Reproduced with permission)

Roggero et al. (2007) suggested a model for acrosomal exocytosis that involved the introduction of extracellular calcium between the acrosome and plasma membrane. This, in turn, was proposed to form loose trans-SNARE complexes, and the intra-acrosomal calcium efflux was thought to trigger the SNARE-mediated acrosome reaction. In the light of their own results, however, Tsai et al. (2010) have refined this model, proposing the following features:

1. The acrosome is not associated with the plasma membrane in uncapacitated spermatozoa.
2. Sperm capacitation leads to stable docking of the outer acrosome membrane to plasma membrane at the apical region of the sperm head without the fusion of both membranes.

3. ZP-binding allows calcium to enter into the sperm (Arnoult et al. 1996; Breitbart 2002). The high levels of cytosolic calcium will induce a conformational calcium-dependent change from trans- to cis-SNARE complex. Then, the two membranes are fused and acrosome reaction occurs (Fig. 7.5).

We can thus conclude that sperm plasmalemma and the outer acrosome membrane become more unstable during late capacitation and stable dock between one another. However, actual fusion between these two membranes only takes place during acrosome reaction, i.e. after sperm bind to ZP. For this reason, this aspect will be specifically treated in the next chapter.

7.7.4 Tyrosine Phosphorylation of Sperm Proteins

Sperm capacitation is also associated with an increase in tyrosine phosphorylation of some sperm proteins (Töpfer-Petersen et al. 2002; Visconti 2009). This phenomenon is a late event that relies on the presence of BSA, Ca^{2+} and bicarbonate (Morgan et al. 2008). Specifically, the absence of any one of these media constituents prevents both tyrosine phosphorylation and capacitation.

Petrunkina et al. (2004), co-incubating spermatozoa with oviductal explants, observed an increase in the tyrosine phosphorylation of tail proteins and later of sperm head proteins in unattached sperm populations during sperm capacitation. In contrast, Töpfer-Petersen et al. (2002) reported spermatozoa bound to oviductal epithelium presented lower calcium uptake and their proteins do not become phosphorylated.

The increase in tyrosine phosphorylation residues of sperm proteins during capacitation is mediated by PKA within in a pathway that is unique to spermatozoa (Signorelli et al. 2012), and has been observed in different mammalian species (Bailey 2010). The involvement of PKA is illustrated by experiments demonstrating that cAMP-permeable analogues are able to induce the increase in tyrosine phosphorylation in the absence of BSA, bicarbonate or calcium.

To date, different substrate-proteins for tyrosine phosphorylation have been identified in mammalian spermatozoa. These substrates are ion channels, enzymes, structural proteins, members of the extracellular signal-regulated kinase family (ERK) family and a calcium-binding protein localised in the principal piece of the tail and associated with the fibrous sheath (Ficarro et al. 2003; Luconi et al. 1998a, b; Naaby-Hansen et al. 2002). One of the main Tyr-phosphorylated structural proteins is a member of the A-kinase-anchoring protein (AKAP) family (Ficarro et al. 2003). These proteins are located at the fibrous sheath and are involved in sperm motility (Muratori et al. 2011).

It is worth noting that phosphorylation of tyrosine residues in sperm proteins does require the presence of bicarbonate and BSA in the surrounding environment, but not that of extracellular calcium, as studies conducted on human spermatozoa have shown (Muratori et al. 2011). In addition, the presence/absence of cholesterol in the sperm membrane is related to the ability of sperm to undergo tyrosine phosphorylation of capacitation-involved proteins. Accordingly, Shadan et al. (2004)

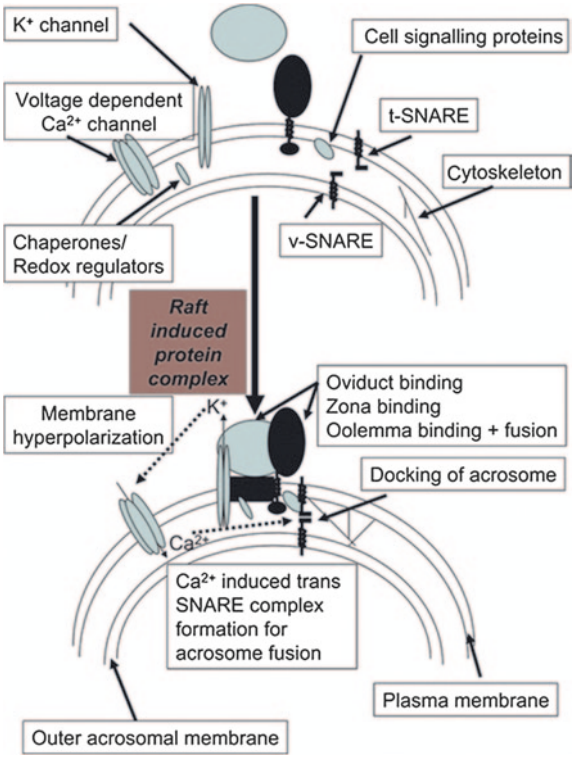

Fig. 7.5 Hypothetical assembly of a sperm-zona binding complex formed during sperm capacitation by raft induced protein clustering. This may result in a multifunctional protein complex known to play a role in diverse processes leading to fertilisation. (Gadella 2008b; Reproduced with permission)

working with boar spermatozoa observed that cholesterol removal from sperm plasmalemma promoted tyrosine phosphorylation of sperm proteins, and Buffone et al. (2009) working with human spermatozoa observed that high cholesterol levels and decreased fluidity of plasma membrane of spermatozoa coming from subfertile/infertile men appeared to be related to deficiencies in tyrosine phosphorylation of such proteins.

In short, the increase in phosphorylated tyrosine residues in sperm proteins is considered to be a marker of late capacitation events (Arcelay et al. 2008). In humans, the relevance of Tyr phosphorylation in sperm capacitation has been demonstrated because it increases the ability of spermatozoa to bind ZP (Liu et al. 2006).

7.8 Bicarbonate, Soluble Adenylyl-Cyclase and cAMP as the Key Mediators of Early/Fast and Late/Slow Sperm Capacitation Events

One of the regulation paradoxes in fast and slow capacitation-associated events is that both are mediated by the same molecules (bicarbonate, sACY and cAMP) and the same transduction pathway (PKA). Thus, although we have separated

fast/early from slow/late capacitation events, the aforementioned three molecules appear, in both cases, as the key molecules involved in their regulation (Esposito et al. 2004; Harrison 2004; Harrison and Miller 2000; Hess et al. 2005; Salicioni et al. 2007; Visconti 2009). Backing this, Morgan et al. (2008), working with mice that bear a mutation in the catalytic subunit of PKA, demonstrated that both fast/early and slow/late capacitation events were activated by the same pathways. When inhibiting this PKA in mutant mice, these authors blocked:

1. The increase in the HCO_3^--dependent flagellar frequency,
2. The phosphorylation of PKA substrates that occurs within 90 s of bicarbonate addition, and
3. The increase in Tyr phosphorylation.

These data led these authors to conclude that PKA has, at least, two independent roles in the regulation of sperm movement:

a. a 'quick' action that is required for the activation of the flagellar beat, and
b. a 'slow' action that allows changes in the pattern of movement of the sperm and that requires PKA to remain active for an extended period of time.

Thus, Morgan et al. (2008) have been the first in proposing a chemical-genetic switch approach to understanding the temporal action of this enzyme in sperm capacitation. However, and despite this ground-breaking study, little is known about how PKA-activity specifically mediates different aspects of sperm capacitation. In this regard, it must be mentioned that although activation of the PKA-pathway occurs immediately and does not need cholesterol acceptors, the increase in tyrosine phosphorylation and other late events are not immediately stimulated and require the presence of cholesterol acceptors (Visconti 2009).

7.9 Phosphorylation/Dephosphorylation of Sperm Proteins During Capacitation

7.9.1 Introduction

As stated, one of the most important changes that takes place during capacitation is the phosphorylation of some sperm proteins. This does not only involve tyrosine residues but also the serine and threonine residues of sperm proteins.

It is worth remembering that mature spermatozoa are transcriptionally quiescent and protein phosphorylation–dephosphorylation thus appears as a method for controlling protein function (Naz and Rajesh 2004; Urner and Sakkas 2003). In addition, it is widely known that regulation of cellular processes requires the coordinated action between kinases and phosphatases. In fact, in the case of mammalian sperm capacitation, 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, recent evidence indicates that phosphatases also play a significant role (Signorelli et al. 2012).

Therefore, and considering the relevance of protein phosphorylation–dephosphorylation in sperm capacitation, the present section exclusively focuses on this aspect and thus reviews the involvement of kinases and phosphatases during this process.

7.9.2 Phosphorylation of Tyrosine Residues in Sperm Proteins

7.9.2.1 Tyrosine Kinases

Tyrosine phosphorylation is mediated by tyrosine kinases, which are activated, directly or indirectly, by PKA (Signorelli et al. 2012). Tyrosine kinases can be divided into two classes:

– Receptor tyrosine kinases (RTKs)
– Non-receptor tyrosine kinases (PTKs).

The first class, RTKs, are transmembrane proteins with an extracellular binding domain to a ligand and an intracellular domain, while the second, PTKs, are found in the cytoplasm, nucleus, or the inner side of the plasma membrane (Fisher et al. 1998). Data on boar spermatozoa are scarce, so that in this subsection we follow currently available studies, which are mainly conducted on human and mouse spermatozoa.

Previous reports have shown the involvement of PTKs in the capacitation of human and mouse spermatozoa. Specifically, four members of the Src-family, *SRC*, *FYN*, *LYN* and *YES1* (*V-YES-1*; Yamaguchi Sarcoma Viral Oncogene Homologue 1) seem to play a relevant role in this regard, the first three members being mainly located in the sperm tail while the other is present in the head. Indeed, YES 1 has been found in the head of human sperm and its activity seems to be modulated by cAMP (Leclerc and Goupil 2002).

Although SRC is also present in the sperm head, only the form located in the mitochondrial/middle piece is active. In humans, SRC presents sites that are phosphorylated by PKA and the phosphorylation of Tyr_{416} leads to its activation during sperm capacitation. This activation leads to acrosome reaction but does not affect sperm motility (Varano et al. 2008). This is because, despite inhibition of PKA and SRC leading to a significant decrease in Tyr phosphorylation, only the inhibition of PKA, but not that of SRC, suppresses sperm motility (Mitchell et al. 2008). In fact, it seems that SRC is involved in the response to calcium triggered by progesterone (Varano et al. 2008).

SRC has also been found in the tail of mouse spermatozoa (Baker et al. 2006; Krapf et al. 2010) and it has also been suggested as a mediator of sperm capacitation in this species (Signorelli et al. 2012).

7.9.2.2 PKA, PKC and ERK/MAPK Pathway

PKA is a tetrameric enzyme that, in somatic cells, contains two regulatory subunits and two catalytic subunits. The activity of this enzyme relies on cAMP, since binding of cAMP to the regulatory subunits leads to tetramer dissociation and allows the activation of the catalytic subunit (Nolan et al. 2004). In the case of spermatozoa, PKA, located on the acrosomal region and in the flagellum, only presents a catalytic (α) subunit (i.e., $C_{\alpha 2}$) (Pariset and Weinman 1994; Visconti et al. 1995a). The critical role of this catalytic subunit in tyrosine phosphorylation and sperm capacitation has been reported, amongst others, by Visconti et al. (1995a) and Esposito et al. (2004). In the first case, inhibition of PKA also resulted in an inhibition of Tyr phosphorylation and sperm capacitation. Furthermore, Esposito et al. (2004), working with Cα2-null mice spermatozoa, observed that capacitating conditions did not induce Tyr phosphorylation and were infertile.

Another protein kinase that has also been found in mammalian spermatozoa, and for which a role in sperm motility and acrosome reaction has been suggested is the calcium-dependent protein kinase (PKC) (Breitbart and Naor 1999).

Apart from PKA, Awda and Buhr (2010) have recently identified three elements (RAF, MEK1/2, and ERK1/2) of the ERK/MAPK pathway in boar spermatozoa, which appears to regulate the phosphorylation of tyrosine residues of different sperm proteins. Awda and Buhr (2010) also observed other kinases from other pathways, like cAMP/PKA and PKC that seem to interact with the MAPK/ERK pathway.

In addition, ROS species play some role in regulating boar sperm capacitation and tyrosine phosphorylation. Awda and Buhr (2010) observed that the presence of ROS inhibited the tyrosine phosphorylation of high-molecular mass proteins, which are phosphorylated during sperm capacitation. According to these authors, inhibition could be explained by interactions with some elements of the MAPKs signal transduction cascade, affecting the phosphorylation of RAF1, MEK1/2, and ERK1/2. On the other hand, Awda and Buhr (2010) observed that the ERK1/2 element of the MAPK/ERK signal transduction pathway is significantly associated with tyrosin-phosphorylation of sperm proteins that takes place during capacitation.

7.9.3 Phosphorylation of Serine/Threonine Residues of Sperm Protein and Ser/Thr Kinases

Apart from the role of Tyrosine kinases and PKA, there are two other kinases that phosphorylate serine and threonine kinases and whose role has also been reported in mammalian species (Fernández-Novell et al. 2011). However, there are fewer reports dealing with this Ser/Thr than with Tyr phosphorylation (Signorelli et al. 2012).

The involvement of Ser and Thr protein phosphorylation in sperm capacitation is quite recent, as it was Naz (1999) who, working with human spermatozoa,

observed that at least four groups of proteins (43–55, 94, 110 and 190 kDa) were phosphorylated on serine residues and threonine residues, during capacitation and after exposure to ZP. Some of these proteins were also observed to be phosphorylated at Tyr residues.

Given the simultaneity of Ser, Thr and Tyr residues on the same proteins, Bedu-Addo et al. (2005), still working with human sperm, observed that phosphorylation on Ser/Thr residues took place earlier than phosphorylation on Tyr residues. This finding provided really novel information about capacitation pathways and about which residues phosphorylate and the chronology of these phosphorylations. In addition, these phosphorylation events were reversible and dependent on the presence of BSA and bicarbonate. In humans, Bedu-Addo et al. (2005) observed that sperm incubated in the absence of both bicarbonate and BSA showed extremely low levels of phosphorylation. In boars, Harrison (2004) observed that bicarbonate stimulation in the absence of BSA led to reduced Ser/Thr phosphorylation levels and lower levels of cellular cAMP than after stimulation of bicarbonate and BSA.

For the time being, and from the available studies, we can state that the dynamics of phosphorylation on Ser and Thr residues during capacitation is very complex, and that we are not currently able to describe an exact picture of what path this phosphorylation follows in mammalian spermatozoa. For instance, and focusing on boar spermatozoa, results are not clear. On one hand, Harayama and Nakamura (2008) have reported a large group of proteins that are phosphorylated on their Ser/Thr residues during capacitation. 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. In addition, Harrison (2004) observed that phosphorylation of Ser/Thr residues of boar sperm proteins significantly increases when spermatozoa are incubated with calyculin-A, a Ser/Thr phosphatase inhibitor. On the other hand, Alnagar et al. (2010) observed dephosphorylation of five phosphorylated proteins in Ser/Thr residues within 15 min of capacitation. However, when spermatozoa are incubated with calyculin-A during sperm capacitation, such dephosphorylation is not observed.

All these data, together with data from Kong et al. (2009) obtained in human spermatozoa, suggest that the proteasome may be involved in the Ser and Thr phosphorylation that takes place during capacitation but not in Tyr phosphorylation.

7.9.4 Protein Phosphatases

As in the case of tyrosine kinases and serine and threonine kinases, more available information about protein kinases exists than about phosphatases. Phosphatases are classified into two groups depending on the residues they dephosphorylate, i.e. protein tyrosine phosphatases that dephosphorylate at Tyr residues, and protein Ser/Thr phosphatase that dephosphorylate Ser and/or Thr residues.

7.9.4.1 Protein Tyrosine Phosphatases

Tyrosine phosphatases are encoded by a large family of phosphatase genes and are divided into three classes (Stoker 2005; Tonks 2006):

- The classical phosphotyrosine (pTyr)-specific phosphatases, which include transmembrane receptor-like proteins and nontransmembrane cytoplasmic protein tyrosine phosphatases.
- Dual-specificity phosphatases (DSPs) and,
- The low-molecular-weight tyrosine phosphatases.

Transmembrane receptor-like proteins are associated with the plasma membrane and regulate signalling through ligand-activated dephosphorylation. These phosphatases are involved in the processes of cellular adhesion and communication. The non-transmembrane cytoplasmic tyrosine phosphatases PTPs are located in various subcellular compartments, such as the cytosol, plasma membrane and the endoplasmic reticulum and do not present transmembrane segments. Some of these phosphatases are also involved in mechanisms of cell adhesion and motility (Zhang et al. 2004).

Another type of tyrosine phosphatases are DSPs. These proteins present smaller catalytic domains than the classical tyrosine phosphatases and are called 'dual' because their activation site accommodates phosphoserine (pSer)/phosphothreonine (pThr) residues and pTyr residues in proteins. Within this class, we find mitogen-activated protein kinase (MAPK) phosphatases (MKPs). These phosphatases inactivate MAPKs through dephosphorylating tyrosine and threonine phosphorylation sites, and participate in MAPK-dependent signalling pathways (Kondoh and Nishida 2007; Tonks 2006). We must remember here that Awda and Buhr (2010) reported that cAMP/PKA and PKC interact with the MAPK/ERK pathway. Another member of this class, VHY, has been found in spermatocytes and in spermatids but not in mature spermatozoa (Alonso et al. 2004).

Finally, it is worth noting that some MKPs are degraded by the ubiquitin–proteasome pathway (Choi et al. 2006). For this reason, the proteasome pathway has been suggested to degrade sperm protein phosphatases (Signorelli et al. 2012).

7.9.4.2 Serine/Threonine Phosphatases

Three different gene families encoding Ser/Thr specific have been reported so far:

1. Protein phosphatases depending on Mg^{2+} or Mn^{2+} (PPM) (e.g. pyruvate dehydrogenase) (Barford et al. 1998)
2. Transcription-factor-IIF-associating C-terminal domain phosphatases (FCP) (e.g. FCP1 and small C-terminal domain phosphatases 1–3) (Gallego and Virshup 2005)
3. Phosphoprotein phosphatases (PPP) that share high homology in the catalytic domains but differ in their N- and C-terminal domains (Barford et al. 1998; Fardilha et al. 2011).

Interestingly, PPP bind to a variety of substrates, are found in various cell types and are involved in the regulation of many cellular functions (e.g. PP1 and PP2 are involved in regulation of the metabolism, cell cycle, cell signalling, muscle contraction, translation or apoptosis) (Cohen 1989, 2002; Millward et al. 1999; Janssens and Goris 2001).

PP4 has been found in the centrosomes of mammals (Sumiyoshi et al. 2002), and PP6 has been detected in the testis of men and bulls (Bastians and Ponstingl 1996).

7.9.4.3 The Specific Case of Phosphatases in Mammalian Spermatozoa

Sperm phosphatases play an important role in the acquisition of motility during sperm maturation (Chakrabarti et al. 2007a; Mishra et al. 2003) and in the acquisition of hyperactivated motility (Fardilha et al. 2011; Krapf et al. 2010) after ejaculation. However, in contrast to the vast knowledge about the involvement of protein kinases in sperm capacitation, little is known about whether protein phosphatases play any role during this process. Thus, even though it is well known that PKA-activation during capacitation leads to phosphorylation of Ser/Thr residues in sperm proteins, it has also been reported that regulating the phosphorylated state of these proteins also involves the participation of Ser/Thr phosphatases.

One of these Ser/Thr phosphatases is the calcium/calmodulin-dependent phosphatase, or calcineurin (PP2B), which was first identified at the postacrosomal region and tail of the spermatozoa of boar, dog, goat, mouse, bovine and sea urchin and is involved in the acquisition of sperm motility (Tash et al. 1988; Tash and Bracho 1994; Carrera et al. 1996). In addition, another phosphatase that has been identified in bovine and fowl spermatozoa is PP2A (Ashizawa et al. 2006; Vijayaraghavan et al. 1996). Despite the role of PP2B and PP2A still remaining unclear in sperm capacitation, Signorelli et al. (2011) recently suggested that, at least in humans, PP2A and PP2B may be involved in sperm capacitation (Signorelli et al. 2011).

Other studies have shown that another Ser/Thr phosphatase, known as PP1, is involved in sperm motility (Mishra et al. 2003). Specifically, this phosphatase has four isoforms of its catalytic subunit (PP1α, PP1β, PP1γ1 and PP1γ2), each one encoded by an individual gene. In the case of spermatozoa, Smith et al. (1996) were the first to observe that PP1γ2 was the main PP1 isoform in human sperm. Thereafter, other studies found PP1γ2 isoform was also present in mice, hamsters, and bulls (Chakrabarti et al. 2007b; Han et al. 2007).

In humans, PP1 is involved in sperm motility and capacitation, whereas PP2A only participates in capacitation, and PP2B regulates sperm motility and hyperactivation (Signorelli et al. 2012).

7.9.4.4 The Specific Case of Phosphatases in Boar Spermatozoa

Focusing on boar spermatozoa, we must say that data are quite scarce and the results that are available at the moment are very recent. Harayama and Nakamura (2008) conducted interesting experiments that compared the response in sperm

capacitation when spermatozoa were incubated with cBIMPS (a cAMP analogue), or with calyculin-A, which is a Ser/Thr phosphatase inhibitor. These authors observed an increase in the percentage of capacitated spermatozoa after incubation with cBIMPS, but not after incubation with calyculin-A.

In addition, Harayama (2003) also found that non-capacitated sperm incubated with cBIMPS presented Ser/Thr-phosphorylated proteins in the postacrosomal region. However, Ser-/Thr-phosphorylated proteins decreased at the postacrosomal region during capacitation, but increased towards the tail (Harayama 2003). In contrast, no changes in the fluorescence patterns associated to Ser-/Thr-phosphorylated proteins were observed in the postacrosomal regions and in the tail after incubation with calyculin-A.

Other reports have also demonstrated that one of the proteins located at the postacrosomal region of non-capacitated spermatozoa, which is phosphorylated when it is in an inactive state, is PP1. In addition, in the presence of cBIMPS, but not in that of calyculin-A, the rate of acrosome-exocytosed spermatozoa increases (about 50 %) (Adachi et al. 2008). Therefore, PP1 seems to play a critical role in boar spermatozoa by dephosphorylating proteins at the postacrosomal region. Specifically, it has been suggested that PP1 mainly suppresses premature acrosome reaction before and after ejaculation, and it is not directly involved in sperm capacitation.

7.10 The Role of the Ubiquitin–Proteasome System in Sperm Capacitation

7.10.1 Ubiquitin

The ubiquitin–proteasome system is the current mechanism of degradation in the most normal and abnormal intracellular proteins (Hochstrasser 1996; Goldberg 2003). In this mechanism, proteins 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. Polyubiquitin chains are assembled via an isopeptidic linkage between a lysine residue of the previous ubiquitin and the C-terminal Gly residue of the subsequent ubiquitin. Various multiubiquitin chains can be formed due to the presence of seven lysine residues in the ubiquitin molecule (Bedford et al. 2010). Chains of four or more ubiquitin moieties linked via Lys_{48} of ubiquitin are known to represent the usual signal for proteasome-mediated proteolysis (Hicke and Dunn 2003).

It is worth noting that the ubiquitination process is balanced with the process of deubiquitination, which is mediated by a number of enzymes. Once marked by polyubiquitin chains, proteins are rapidly degraded by the 26S proteasome. In addition, deregulation of ubiquitination/deubiquitination has been related to diseases in humans (Zhang et al. 2007a), and to male infertility (Martínez-Heredia et

al. 2008; Siva et al. 2010), since regulated ubiquitination marks sperm mitochondria for destruction in a current fertilisation process (see also Sect. 8.9).

Finally, we must also mention that ubiquitin also tags defective sperm during epididymal passage, in a mechanism that seems to mark the abnormal spermatozoa for proteolytic destruction (Sutovsky et al. 2001). This can be regarded as an ubiquitin-dependent sperm quality control that resides in the somatic cells of epididymal epithelium (Tengowski et al. 2007) and detects spermatozoa with fragmented DNA or with other defects (Sutovsky et al. 2002). Matching with this, increased sperm ubiquitin has been inversely associated with sperm concentration, motility and morphology in humans (Sutovsky et al. 2004a).

7.10.2 The Proteasome and its Regulation

The proteasome is involved in the degradation of the majority of intracellular proteins. Proteolytically active sites are within the 20S core particle, which is a cylindrically shaped structure formed by four stacked rings in a α7β7β7α7 pattern (Ciechanover 1998, 2005a, b, 2006; Ciechanover and Schwartz 1998). Subunits β1, β2 and β5 have proteolytic activity and display trypsin-like, chymotrypsin-like and caspase-like peptidase activity, respectively (Groll et al. 2001) (Fig. 7.6).

Apart from the core particle, proteasome presents a regulatory particle that contains six ATPases. This is important, since protein degradation is an ATP-dependent mechanism that needs the presence of ATP-hydrolysing proteins (Bedford et al. 2010). Although core particles can associate with one of two regulatory particles, there are also other activators that can bind the core particle-20S (Gallastegui and Groll 2010).

The function of 26S proteasome is modulated by phosphorylation/dephosphorylation of such ATPases, in a process that is mediated by PKA and phosphatases. Accordingly, phosphorylation of the ATPase subunits by PKA increases the chymotryptic and tryptic activity of proteasome and is reversed by phosphatase 1γ (PP1γ) (Zhang et al. 2007b). Working with cardiac proteasomes in murine species, Zong et al. (2006) observed an increase in their proteolytic activity after activation of PKA or inhibition of another phosphatase PP2A.

As stated above, tyrosine kinases (PKA) and phosphatases (PP1γ and PP2A) have been found in mammalian spermatozoa and phosphorylation, mediated by PKA and these phosphatases appear as a key mechanism for regulating the proteasome function. This background has led Signorelli et al. (2012) to suggest that the cAMP/PKA pathway regulates proteasome phosphorylation/activity in spermatozoa.

7.10.3 Phosphorylation and Proteasomes

The phosphorylation of a given protein can be a signal for its further degradation via the ubiquitin–proteasome pathway (Glickman and Ciechanover 2002;

Tanaka and Chiba 1998). Related to this, phosphatases have been reported to be targeted by the ubiquitin–proteasome system in somatic cells (Brush and Shenolikar 2008; Trockenbacher et al. 2001).

In the case of spermatozoa, Signorelli et al. (2012) have mentioned the relationship between protein phosphorylation and the proteasomal activity may also be interesting, for two reasons:

1. Proteasomes have been found in mammalian spermatozoa and they play a relevant role during fertilisation as discussed in the next Chapter (Morales et al. 2003, 2004, 2007; Sutovsky et al. 2000, 2004b; Zimmerman and Sutovsky 2009; Zimmerman et al. 2011).
2. Phosphorylation of some proteasome subunits exerts a significant regulatory role (Bose et al. 1999; Pardo et al. 1998; Rivett et al. 2001).

7.10.4 The Case of Sperm Capacitation

After identification of the sperm proteome, the ubiquitin–proteasome system has been reported to be involved in both capacitation and fertilisation (Baker et al. 2010; Sutovsky 2011; Zimmerman and Sutovsky 2009; see also Chap. 8). Indeed, proteasomes, which are protein complexes, have been located in the plasma membrane of the sperm head and neck in pig (Yi et al. 2007), human (Tipler et al. 1997; Wojcik et al. 2000; Morales et al. 2004), mouse (Pasten et al. 2005) and ascidian (Sawada et al. 2002) spermatozoa, and a function for this proteasome pool has been suggested by Yi et al. (2007) and Zimmerman et al. (2011).

As mentioned before, protein phosphorylation both on Ser/Thr and on Tyr residues is a hallmark of sperm capacitation (Bailey 2010). Related to this, proteins belonging to the ubiquitin–proteasome system have been seen to phosphorylate during the capacitation of boar, mouse and rat spermatozoa (Arcelay et al. 2008; Baker et al. 2010). These proteins were: the ubiquitin itself (Arcelay et al. 2008), the ubiquitin activating enzyme UBE (Baker et al. 2010), multiple proteasomal subunits (Arcelay et al. 2008; Baker et al. 2010), and the valosine-containing protein, which is involved in the ubiquitinated substrate presentation to the 26S proteasome (Geussova et al. 2002). In addition, Choi et al. (2008) observed differences in the electrophoretic patterns depicted by several 20S core subunits in capacitated and non-capacitated boar spermatozoa. Therefore, growing evidence clearly indicates that sperm proteasomes are involved in sperm capacitation.

In functional studies conducted with human spermatozoa, it was seen that when sperm became capacitated, the chymotrypsin-like activity of their 20S proteasomal core increased, and an increase of the phosphorylation of several proteasomal subunits both on Ser/Thr and on Tyr residues also took place (Kong et al. 2009). However, when inhibitors of ser/Thr and of Tyr kinases (PKA and PKC) were added to capacitation medium, the chymotrypsin-like activity of 20S diminished (Kong et al. 2009). Other complementary studies, also performed in human sperm,

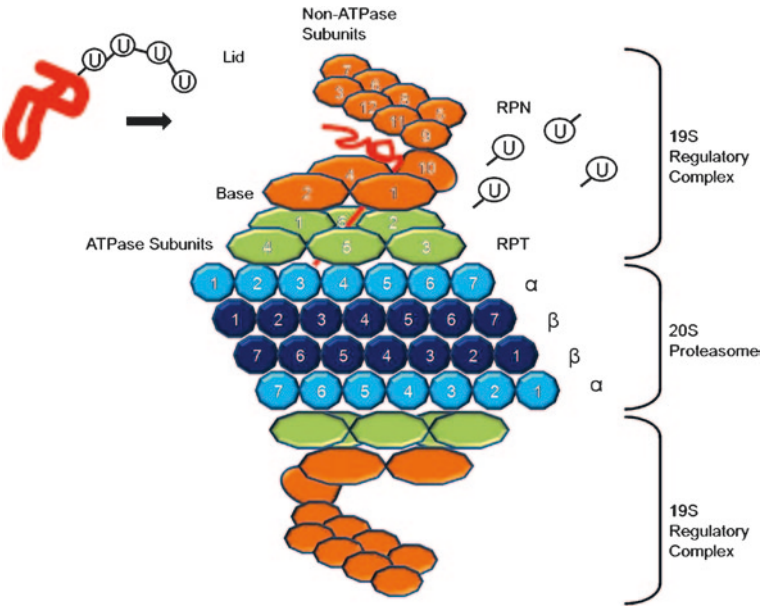

Fig. 7.6 Drawing of the 26S proteasome. This protein complex consists of the 19S regulatory complex and the 20S core, which contains two α- and two β-rings, each made of 7 subunits. On either side of the core resides there is a 19S regulatory complex; this complex recognises proteins bound to multiubiquitin chains, removes the multiubiquitin chains and primes the substrate proteins for degradation. Recognition of ubiquitinated proteins occurs primarily through subunit RPN10; upon recognition the ubiquitin molecules are released through the recruitment of deubiquitinating enzymes to the 19S complex, and the deubiquitinated protein is unfolded and transported into the 20S core (Zimmerman and Sutovsky 2009; Reproduced with permission)

demonstrated that epoxomicin, an inhibitor of proteasome activity, reduced Ser phosphorylation during sperm capacitation (Morales et al. 2007). Therefore, inhibitors of tyrosine kinases and PKA decrease the activity of the proteasome during capacitation, and proteasomes are phosphorylated during capacitation via tyrosine kinases and PKA (Kong et al. 2009; Morales et al. 2007).

According to Sutovsky (2011), all these findings open up the possibility that, although regulated itself by phosphorylation, proteasomal activity may directly or indirectly govern the activity of sperm-borne protein kinases. These events might not only occur at the sperm acrosome but also in the principal piece of the sperm tail, where proteasomes could exert an effect on the capacitation-related changes of sperm motility.

In short, it is quite clear that sperm proteasomes play an active role in the capacitation process and that proteasome activity is modulated by protein kinases. However, more research on the role of the ubiquitin–proteasome system in sperm capacitation is warranted since there are still several relevant aspects of this process that remain to be elucidated.

7.11 Regulation Mechanisms of Sperm Capacitation

7.11.1 Defective Regulation of Sperm Capacitation In Vivo

One of the most important aspects of the regulation of sperm capacitation within the oviduct is that spermatozoa have to remain uncapacitated and acrosome intact until they meet the egg. This crucial and well-coordinated event has been extensively described in the previous Chapter, when speaking about the importance of the sperm reservoir in mammalian and, thus, in swine reproduction (see Chap. 6).

In vivo, there are ligands that interact with G-protein coupled receptors (GPCRs) and inhibit the membrane-associated adenylyl-cyclases. This reduces the production of cAMP production in capacitated spermatozoa and prevents spontaneous/degenerative acrosome reactions (Fraser 2010). These ligands that inhibit spontaneous acrosome reaction have been found in mouse (Fraser et al. 2006), in boar (Funahashi et al. 2000a, b) and human (Fraser and Osiguwa 2004) spermatozoa. However, this inhibition of spontaneous acrosome loss does not prevent spermatozoa from undergoing acrosome reactions in response to either progesterone (Green et al. 1996) or the zona intact-oocytes (Fraser et al. 1997; Funahashi et al. 2000b).

Finally, it is worth mentioning that defective regulation of sperm capacitation may be related to infertility. Thus, in human sperm, Fraser and Osiguwa (2004) have observed higher percentages of capacitated and acrosome-reacted spermatozoa in infertile than in fertile men. This finding suggests that spermatozoa from infertile men may undergo accelerated capacitation and hence die before reaching an oocyte. Different causes could explain this phenomenon, such as a lack of decapacitation factors, lack of GPCR-modulators, or problems with GPCRs themselves (Fraser 2010).

7.11.2 The Role of 'Decapacitation' Factors

7.11.2.1 Introduction

One of the main disadvantages of studying sperm capacitation and fertilisation is that information about these crucial events comes from in vitro rather than in vivo studies. In vitro, some spermatozoa can undergo a degenerative/spontaneous acrosome reaction if damages like cryopreservation, short/cold shocks, or other environmental changes are infringed. However, acrosome-reacted spermatozoa have lost their fertilising ability, because they do not present the anterior plasma membrane that has the molecules needed for interacting with ZP-glycoproteins (Fraser 2010).

However, some aspects that avoid premature/degenerative acrosome reaction must be considered in vivo. Thus, despite spermatozoa not having intrinsic mechanisms to put a brake on this 'over-capacitation', seminal plasma and female tract

secretions contain several molecules that can interact with external receptors of sperm surface and inhibit spontaneous acrosome exocytosis (Fraser et al. 2003). This emphasises the relevance of spermatozoa keeping their fertilising ability by retaining intact their acrosomes while awaiting the oocyte (Fraser 2010).

Apart from the early sACY-dependent changes in sperm plasma membrane architecture, there are other changes that involve the loss, unmasking, or rearrangement of molecules on the sperm surface (Fraser 2010). Entities that are lost during process are usually referred to as 'decapacitation factors' (Bedford and Chang 1962; de Lamirande et al. 1997), because their addition to capacitated spermatozoa may reverse capacitation, causing cells to become 'decapacitated', i.e. they revert sperm to the non-fertilising state. With time, spermatozoa can recapacitate and regain fertility, indicating that capacitation itself is 'reversible'. Despite this, care must be taken when talking about 'decapacitation' and 'reversibility', since this often refers to early-capacitation events, and because some steps of this process, like membrane docking, are not reversible. Thus, it is worth noting that when capacitation is reversed by decapacitation factors there is no back addition of cholesterol to the plasma membrane, even though spermatozoa do not present fertilising ability (Fraser 2010).

To date, most of the knowledge about decapacitation factors has come from epididymal mouse spermatozoa. Indeed, Fraser (1984) reported more than two decades ago that when decapacitation factors were added to capacitating/capacitated spermatozoa, there was an inhibition of the sperm's fertilising ability. Spermatozoa could, however, regain fertilising ability when they were reincubated in a capacitation medium that did not contain decapacitation factors (Fraser 1984). Notwithstanding, DasGupta et al. (1994) observed that mouse decapacitation factors were also able to reverse the proportion of capacitated to uncapacitated status in human spermatozoa.

7.11.2.2 The Nature of Decapacitation Factors and Their Specific Receptors

According to Fraser et al. (1990), the decapacitation factor (DF) in mice is an anionic protein of about 40 kDa and stable to heating and proteolytic degradation. This molecule contains fucose residues, which are critical for the function that this factor exerts (Fraser 1998a). In fact, when fucose is added to a medium containing uncapacitated spermatozoa there is an increase in sperm capacitation and an increase in the sperm's fertilising ability. This has been observed both in mouse (Fraser 1998a) and human spermatozoa (Fraser and Osiguwa 2004).

More recent studies have demonstrated that the DF binds to a specific receptor that has fucose-binding sites and is attached to the sperm surface via a glycosylphosphatidylinositol (GPI) anchor (Fraser 2010). When the DF binds to its specific receptor, calcium ATPase is activated (Fig. 7.7). In contrast, when the DF is lost during capacitation, the activity of this ATPase is reduced and this leads to an increase in intracellular calcium levels (Adeoya-Osiguwa and Fraser 1996).

This explains why amounts of intracellular calcium increase during capacitation (Florman 1995).

A DF receptor (DF-R) presents high homology with phosphatidylethanolamine-binding protein 1 (PEBP 1) (Gibbons et al. 2005). In immunocytochemical studies, PEBP1 has been indentified on the head and the tail of both mouse and human spermatozoa. These studies have also shown that the fluorescence intensity in these regions varies, and this variation depends on the capacitation status of spermatozoa. In addition, these fluorescence patterns also change when decapacitation factors are added, and a reversion from capacitated to uncapacitated spermatozoa has, therefore, taken place (Gibbons et al. 2005).

7.11.2.3 Decapacitation Factors and Capacitation-Related Changes of Sperm Membrane

The presence/absence of decapacitation factors appears to cause conformational changes in the specific receptor (DF-R) of these factors (Fraser 2010). These conformational changes lead to alterations in the functionality of several membrane-associated proteins, such as GPCRs and calcium ATPase (Gibbons et al. 2005) (Fig. 7.7). This has been confirmed in a study conducted with mouse spermatozoa that assessed the role of adenosine receptors (Adeoya-Osiguwa and Fraser 2002). Related to this, we must indicate that mouse spermatozoa have two populations of adenosine receptors (GPCRs), stimulatory $A_{2\text{-}A}$ and inhibitory A_1, and that the function of these two receptors depends on their capacitation status. Thus, while the stimulatory $A_{2\text{-}A}$ is only active in uncapacitated spermatozoa, the inhibitory A_1 receptor only works in capacitated spermatozoa (Fraser and Adeoya-Osiguwa 1999).

Interestingly, Adeoya-Osiguwa and Fraser (2002) observed that when exogenous decapacitation factors are added to capacitated spermatozoa, A_1 receptors become unresponsive to A_1 agonists while $A_{2\text{-}A}$ receptors, previously unresponsive to $A_{2\text{-}A}$ agonists, are reactivated. Thus, when decapacitation factors are added to capacitated spermatozoa and they bind to their own specific receptors, there is an altered accessibility of adenosine binding sites in these receptors.

Other findings that support the idea that interaction of decapacitation factors with their specific receptors affects other membrane proteins are related to the organisation of sperm membrane. As previously mentioned, the membrane lipid microdomains (DRMDs) play a relevant role during docking/fusion between outer acrosome and plasma membrane. We should recall that these DRMDs are enriched in cholesterol and sphingolipids, and contain molecules that appear to mediate signal transduction pathways (Pike 2009; Tsai et al. 2007, 2010).

When spermatozoa are in vitro capacitated, there is a reorganisation of membrane architecture that affects these lipid rafts and the proteins that these DRMDs contain (Cross 2004; Van Gestel et al. 2005). In this regard, it is important to take into account that GPI-anchored proteins are frequently

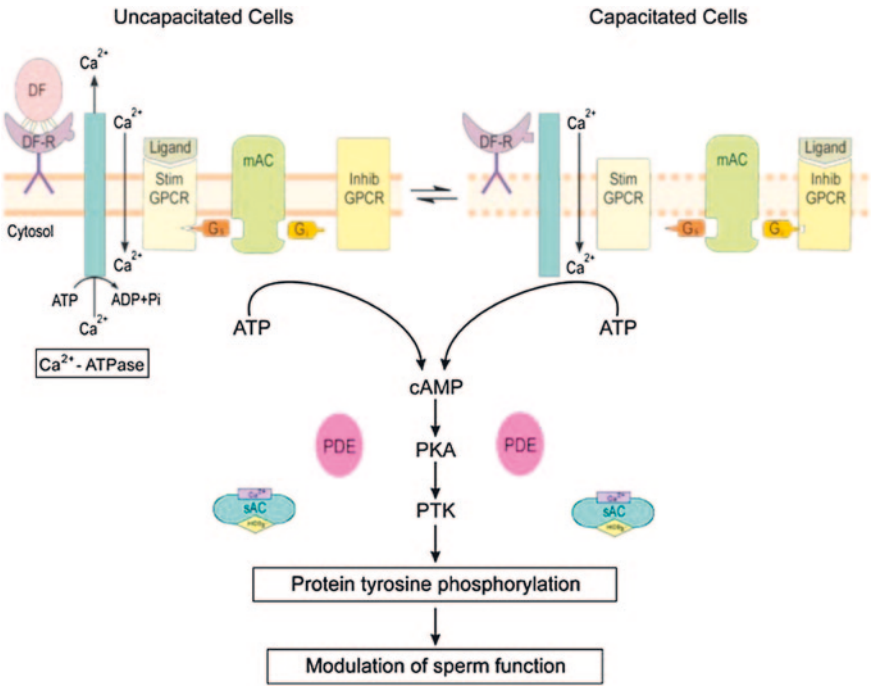

Fig. 7.7 Schematic diagram depicting the proposed mechanism of action of mammalian sperm DF during capacitation, resulting in altered availability of cAMP. The presence or absence of DF bound to its receptor DF-R (PEBP 1) and alters the conformation and function of various membrane-associated proteins, including Ca^{2+}-ATPase and GPCRs. If an appropriate exogenous ligand binds to a GPCR, the receptor will then interact with G_s or G_i to modulate the function of mAC/mACY, either stimulating or inhibiting cAMP production, respectively. In the cytoplasm, there is a sAC/sACY activated by Ca^{2+} and bicarbonate. The availability of cAMP within cells, reflecting the relative activities of mACs, sAC and phosphodiesterases (PDEs), will determine the activity of PKA and protein tyrosine kinase (PTK), which will in turn either stimulate or inhibit protein tyrosine phosphorylation and, thus, modify sperm function (Fraser 2010; Reproduced with permission)

associated with DRMDs (Lai 2003; Mayor and Riezman 2004; Pike 2009), and that a GPI anchor is involved in the attachment of the receptor of DF to the sperm plasma membrane (Gibbons et al. 2005). If we relate these two findings, we may explain the mechanism by which decapacitation factors act. Indeed, if the DF receptor is located in DRMDs and these lipid domains undergo conformation changes during capacitation, it is quite likely that the function of this receptor will be affected during capacitation. This hypothesis is supported by the more recent results provided by Asano et al. (2009). These authors have localised PEBP1/DF-R in DRMDs of sperm head and flagellum, which matches with previously mentioned results obtained in immunolocalisation studies and conducted with mouse and human spermatozoa (Gibbons et al. 2005).

7.11.3 Small Molecules that can Regulate Sperm Capacitation

7.11.3.1 Introduction

After being released from the sperm reservoir, the spermatozoa find an appropriate milieu to continue capacitation up to its completion (Fraser 2008). However, this step might lead some sperm cells to 'over-capacitation', resulting in spontaneous and undesired acrosome reactions, since only spermatozoa with an intact plasma membrane can interact with ZP and overcapacitated spermatozoa lose their fertilising ability. Accordingly, despite being important that spermatozoa become capacitated, the acrosome reaction has to be delayed until sperm do not interact with ZP. This issue will be discussed in the next subsections.

7.11.3.2 FPP, Adenosine and Calcitonin

The role of small molecules in sperm capacitation was first observed in the tripeptide pyroglutamylglutamylprolineamide (pGlu-Glu-ProNH_2) (Fraser 2008), later known as 'fertilisation promoting peptide' (FPP). This molecule is found in the seminal plasma at high concentrations (FPP is produced in the prostate gland), and comes into contact with spermatozoa upon ejaculation. FPP is structurally related to thyrotrophin-releasing hormone (TRH), elicits a biphasic response, since on the one hand it accelerates capacitation in uncapacitated sperm, but at the same time it inhibits spontaneous acrosome reactions in capacitated sperm (Fraser et al. 2003).

Apart from FPP, adenosine, calcitonin, and adrenaline have also been reported to be able to stimulate capacitation and also to inhibit acrosome reaction (Fraser 2008, 2010; Fraser et al. 2006; Adeoya-Osiguwa and Fraser 2005). In in vitro procedures, the addition of adenosine and FPP diminishes polyspermy in mouse and boar spermatozoa, since these two molecules also appear to accelerate sperm capacitation and to reduce the number of spontaneous acrosome reactions (Funahashi et al. 2000a, b).

Interestingly, the biological sperm response of these small molecules depends on their concentration. Thus, high levels of FPP, such as those found immediately after ejaculation, prevent sperm capacitation, but this concentration drops in the female reproductive tract after mixing with vaginal secretions and/or becomes less active due to the vagina-pH (Fraser 1998b, 2008). In addition, the concentration required to inhibit the spontaneous acrosome reaction is higher than that required for accelerating capacitation (Fraser and Adeoya-Osiguwa 1999). All these data also back the idea that these small molecules regulate sperm capacitation and acrosome reaction in a biphasic-manner accelerating capacitation but inhibiting acrosome reaction. The final effect of these molecules is the increase of fertilisation rates along with a decrease in monospermy rates, thereby helping the spermatozoa to maintain their fertilising ability until they reach the oocyte (Fraser 2010).

7.11.3.3 Action Mechanisms of Small Molecules that Regulate Sperm Capacitation

FPP affects cAMP levels, stimulating its production in uncapacitated sperm and inhibiting it in capacitated suspensions (Fraser et al. 2003). The mechanism of action involves the modulation of membrane-associated adenylyl cyclase (mACY) and cAMP production. In other studies, adenosine (Fraser et al. 2003), calcitonin (Fraser et al. 2006) and adrenaline (Adeoya-Osiguwa and Fraser 2005) also trigger the same response, i.e. stimulation of capacitation and fertilising ability, inhibition of spontaneous acrosome reactions, by regulating mACY and cAMP levels.

In the presence of the proper 'first messenger', the mammalian spermatozoon has a number of signal transduction pathways that can modulate the cAMP production (Fraser 2008). In the acrosomal cap region and in the flagellum, the mammalian spermatozoa present specific receptors for these small molecules, i.e. adenosine, calcitonin, adrenaline and FPP (Fraser et al. 2003, 2006; Adeoya-Osiguwa et al. 2006) (Fig. 7.8). Specific receptors for adenosine, calcitonin and adrenaline are GPCRs and are often involved in modulation of mACY activity in somatic cells, while the mechanism of action of the FPP receptor is not exactly known, but it has a synergistic stimulatory effect with adenosine that increases mACY activity in the sperm (Fraser 2010) (Fig. 7.8).

In the case of adenosine receptors (GPCRs), spermatozoa present both stimulatory $A_{2\text{-}A}$ and inhibitory A_1 receptors and work, as stated, in a capacitation state-dependent manner. Thus, stimulatory receptors only work in uncapacitated sperm while the inhibitory receptors only function in capacitated cells (Fraser 2008) (see also Sect. 7.11.3). Similarly, stimulatory β (β_1, β_2, and β_3) and inhibitory α (α_{2A}) adrenergic receptors are also present in the mammalian spermatozoa and their function changes also depend on capacitation status. Moreover, sperm cells only present one type of calcitonin receptor, even though they also function in a capacitation state-dependent manner (Fraser et al. 2003, 2006; Fraser 2008) (Fig. 7.8).

Although some authors considered that mammalian spermatozoa possess inhibitory-Gα subunits ($G\alpha_i$; required for inhibition of mACY-directed cAMP production), but lack stimulatory $G\alpha_s$ subunits ($G\alpha_s$; required for stimulation of mACY-directed cAMP production), biochemical, immunohistochemical and physiological studies have now shown that $G\alpha_s$ subunits are also present (Fraser et al. 2003; Spehr et al. 2004). In addition, cholera toxin enhances ADP-ribosylation of a protein that has a similar size to $G\alpha_s$ (Bentley et al. 1986; Kopf et al. 1986; Baxendale and Fraser 2003). Therefore, the signalling pathway components required for external ligands to activate GPCRs that regulate cAMP production via mACY are present in spermatozoa. Accordingly, stimulatory ($G\alpha_s$, $G\alpha_{olf}$) and inhibitory ($G\alpha_{i2}$) Gα subunits, $mACY_3$ and $mACY_8$ isoforms and several cyclic nucleotide phosphodiesterases (PDEs) have been detected close to the GPCRs (Fraser 2010). Specifically, these components are found both in sperm head and in sperm tail (Fraser et al. 2003; Fraser 2010), since PDE1 has been located in the flagellum and stimulates capacitation, and PDE4 has been identified both in the head and in the flagellum and inhibits capacitation (Fraser et al. 2006).

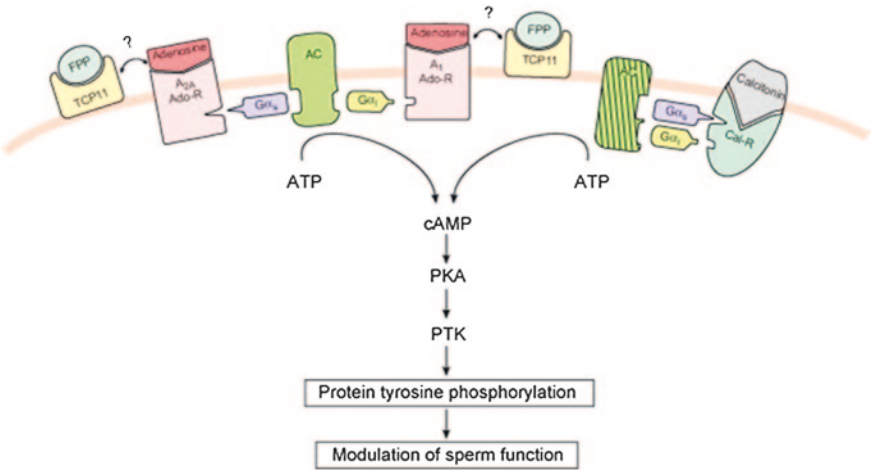

Fig. 7.8 Schematic diagram depicting the signal transduction pathways in sperm regulated by FPP, adenosine and calcitonin. These first messengers interact with their specific receptors to regulate mACY activity and cAMP production during capacitation (Fraser 2008; Reproduced with permission)

If we consider all these data, it appears that mammalian spermatozoa have several signalling pathways able to regulate mACY/cAMP. These pathways are triggered after binding of the specific receptors to the mentioned small molecules, which act as ligands (first messengers). In all cases, it seems that the activated pathway in uncapacitated spermatozoa leads to an increase in cAMP levels which, in turn, accelerates sperm capacitation with respect to the absence of the ligand (Fraser 2008). Later, when spermatozoa have become capacitated, the set of sperm membrane changes related to this process induce conformational alterations in the various receptors, so that the same ligand does not increase cAMP levels, but decreases them. This decrease leads to an inhibition of spontaneous acrosome reaction and ensures the maintenance of sperm fertilising ability, thereby maximising the number of potentially fertilising spermatozoa in the vicinity of unfertilised oocytes. As mentioned before, the first messengers involved in this mechanism would be the small molecules that are present in various body fluids and for which the spermatozoa present specific receptors (Fraser et al. 2006; Fraser 2008).

Finally, it is worth noting that in a possibly wrong approach, caffeine has been usefully added to IVF media to increase the fertilisation rate. However, the main problem of this methylxanthine is that it causes an unregulated cAMP increase by inhibiting PDEs, so that when intracellular cAMP reaches a threshold level, this can trigger spontaneous/degenerative acrosome reactions (Fraser 2010). In contrast, adenosine regulates cAMP and keeps cells acrosome-intact, suggesting that it should be possible to use lower sperm concentrations than those routinely used with caffeine-treated suspensions (Funahashi et al. 2000b). Thus, the addition of adenosine and FPP does not only increase fertilisation rates, like caffeine, but also

the rates of monospermic penetration (Funahashi and Nagai 2001; Funahashi and Romar 2004; Suzuki et al. 2005). This warrants the use of these small molecules to improve the success of assisted reproduction techniques in mammals.

7.11.4 Role of Dopamine Type 2 Receptor in the Modulation of Sperm Capacitation

In the oviduct of humans (Helm et al. 1982), swine (Chaud et al. 1983), rabbits (Khatchadourian et al. 1987), and cattle (Kotwica et al. 2003), there are high levels of catecholamines (the most abundant are adrenaline, noradrenaline and dopamine). These levels of catecholamine vary according to the oviductal region and to the phase of the oestrous cycle, thereby suggesting that they are under hypothalamo-pituitary control (Ramírez et al. 2009).

High levels of catecholamines have also been found in human semen (Fait et al. 2001), and mammalian spermatozoa also present receptors for these neurotransmitters (Meizel 2004). Indeed, male rat germ cells (Otth et al. 2007) and spermatozoa from boar, rat, mouse, human and bull express dopamine type 2 (D2)-like receptors dopamine type 2 receptor (DRD2) (Ramírez et al. 2009). This indicates that the presence of this dopamine receptor is highly conserved in mammalian spermatozoa and also suggests that DRD2 is a potential target for endogenous dopamine. In addition, Ramírez et al. (2009) have also demonstrated that boar, human, mouse, bull and horse spermatozoa have a catecholaminergic phenotype and are a sensitive cellular target for cocaine, amphetamine, and antidepressant and antipsychotic drugs.

One of the most abundant catecholamines in the body of swine and other mammals, together with adrenaline and noradrenaline, is dopamine. This catecholamine is a neurotransmitter within the mammalian central nervous system that has an important role in functions such as cognition, emotion and motor activity control (Aumann and Horne 2012). Alteration of certain elements of the dopaminergic system has been associated with neurological and psychiatric disorders such as Parkinson's disease (Picconi et al. 2012) and schizophrenia (Yin et al. 2012). In addition, dopamine seems to act as a physiological modulator of capacitation, viability and motility of mammalian spermatozoa, as studies conducted in porcine have shown (Ramírez et al. 2009).

Receptors of dopamine are seven-transmembrane trimeric guanosine triphosphate (GTP)-binding protein (G protein)-coupled receptors, classified into distinct subfamilies based on their pharmacological characteristics and their sequence homology. Examples of dopamine are the dopamine type 1 (D1)-like receptors (DRD1 and DRD4) and the D2-like receptors (DRD2, DRD3, and DRD4) (Missale et al. 1998). Stimulation of D1-like receptors activates adenylyl cyclase by coupling to stimulatory GTP-binding regulatory protein (G_s protein). This increases cAMP accumulation and activates the cAMP/PKA pathway (Himmler et al. 1993; Das et al. 1997). In contrast, stimulation of D2-like receptors that

show high affinity for antipsychotic drugs inhibits adenylyl cyclases by coupling to inhibitory GTP-binding regulatory protein ($G_{i/o}$). In this case there is a decrease rather than an increase in PKA activity (Missale et al. 1998).

Catecholamines and phenylpropanolamines, which act as potent and selective releasing agents of adrenaline, noradrenaline and to a lesser extent of dopamine and function as endogenous catecholamines, modulate sperm capacitation and acrosome exocytosis. Indeed, phenylpropanolamines have been found to accelerate capacitation but inhibit spontaneous acrosome reaction in mouse spermatozoa (Adeoya-Osiguwa and Fraser 2005). Catecholamines play a relevant role in the later stages of sperm capacitation, since adrenaline, noradrenaline and isoproterenol increase the number of hamster sperm with hyperactivated motility (Cornett and Meizel 1978) and the proportion of spontaneous acrosomal exocytosis in hamster (Cornett and Meizel 1978; Meizel and Working 1980) and in bull spermatozoa (Way and Killian 2002). The exact role of these catecholamines is, however, not exactly known, since at lower concentrations noradrenaline induces sperm capacitation, but at higher concentrations it prevents capacitation in bull spermatozoa (Way and Killian 2002). Related to this, Schuh et al. (2007) have demonstrated that adenosine (see also Sect. 7.11.3), adrenaline and noradrenaline increase the velocity of the flagellar beat by a non-receptor-mediated mechanism. This means that catecholamines could enter by sodium- and chloride-dependent transport mechanisms. These catecholamines might then inhibit phosphodiesterase that, in turn, would increase the levels of cAMP (Ramírez et al. 2009).

As previously mentioned, changes in tyrosine phosphorylation following increases in cAMP levels and the activation of PKA have been proposed as the key mechanisms by which sperm capacitation and acrosome reaction are regulated (Visconti et al. 1995a, b; Bajpai and Doncel 2003). In the case of boar spermatozoa, Ramírez et al. (2009) have observed that dopamine exerts an effect on sperm capacitation, specifically on tyrosine phosphorylation, but does not induce acrosome loss. Again, however, the role of this catecholamine is dual and depends on its concentration. Thus, at higher concentration, dopamine decreases sperm motility and the tyrosine phosphorylation pattern of sperm proteins, by a transporter-mediated mechanism for catecholamine uptake (Ramírez et al. 2009). Related to this, an excess of products from dopamine oxidation are toxic for sperm and appear to cause the inhibitory effects of dopamine on tyrosine phosphorylation and sperm motility (Ramírez et al. 2009). Conversely, at low concentrations, dopamine increases sperm viability and total and progressive motility at 2 h of capacitation through DRD 2 activation. The activation of DRD increases AKT-phosphorylation at Serine-473 (Ser_{473}) residue as occurs in neurons (Brami-Cherrier et al. 2002; Kihara et al. 2002; Nair and Sealfon 2003; Ramírez et al. 2009). This observation indicates that dopamine seems to play a significant role in maintaining sperm viability at early stages of the capacitation process.

Although it has been reported that the activation of DRD2 entails the reduction of cAMP levels, as has been seen in rat ovaries (Hall and Strange 1999), DRD2 also modulates $G\alpha_s$, a subunit protein that couples the cannabinoid receptor CB1 (Jarrahian et al. 2004), which is present in human (Rossato et al. 2005) and boar

(Maccarrone et al. 2005) spermatozoa. In this regard, Ramírez et al. (2009) have proposed that the modulation of the interaction of DRD2 and CB1 could affect the cAMP levels before and after the onset of sperm capacitation, in a manner that might be independent of the presence of progesterone.

Finally, it is worth noting that the association of DRD2 with tyrosine phosphorylated proteins, which still remain unidentified, increases during sperm capacitation, and this occurs independently from DRD2 activation. In fact, the localisation of both DRD2 and tyrosine phosphorylated proteins depends on the capacitation status (Tardif et al. 2001; Ramírez et al. 2009). Therefore, in uncapacitated spermatozoa, DRD2 is found in the flagella and pTyr proteins are predominantly localised in the post-equatorial area of the head. In capacitated spermatozoa, DRD2 is present in the flagella but also in the acrosomal region of sperm head, whereas the presence of tyrosine phosphorylated proteins increases in the midpiece and the acrosomal region co-localised with DRD2 (Ramírez et al. 2009). All these findings suggest the presence of a signalling complex that contains DRD2 and tyrosine phosphorylation target proteins and seems to play a relevant role in energy control and in the modulation of sperm capacitation and motility, and in acrosome reaction.

7.12 In Silico Studies and the Coordinating Role of Actin in Capacitation Network

To conclude this chapter, we focus now on recent and novel studies dealing with the coordination of the subcellular signalling process during boar sperm capacitation. These studies have used computational models, also known as in silico models, and have later been confirmed by in vitro experiments (Bernabò et al. 2011). From these results, the actin cytoskeleton has been suggested to play a key role in signalling capacitation, apart from mechanically supporting the cell. The actin cytoskeleton appears thus to be a node that coordinates signalling cascades of different intracellular compartments during capacitation.

By means of this model, these authors have also found the functional meaning and the localisation of all the molecules involved in sperm capacitation and the nodes that interconnect the intracellular compartments. They have identified three key nodes: the intracellular levels of calcium, the ATP levels and 'actin polymerisation'. From these three elements, they have observed that two of them (intracellular Ca^{2+} and ATP) were linked with a high number of links, thereby working as ubiquitous second messengers (as in the case of intracellular calcium; 28 links), or as metabolic sustainment (as in the case of ATP; 15 links). The same group previously reported that ablation from the network provokes the collapse of network structures (Bernabò et al. 2010b).

As far as the third node (actin polymerisation) is concerned, Bernabò et al. (2011) have found a lower number of links (eight) relating to all the subcellular compartments involved in capacitation (cytosol, cytoskeleton, mitochondria

and acrosome). In contrast, it is worth noting that these authors have not found actin polymerisation to be related to the sperm nucleus during capacitation. This seems logical since the nucleus is the only compartment of the spermatozoon that remains stable during capacitation. Interestingly, the actin polymerisation links during capacitation have also been indentified and they are the following (Bernabò et al. 2010b):

- Phosphatidic acid (i.e. the product of phospholipase D). This can be explained by the dependence of actin polymerisation on the activation of phospholipase D. This activation takes place via the bicarbonate/cAMP/PKA pathway or via the G-protein coupled receptor (GPCR)/PKC pathway (Breitbart et al. 2005).
- ATP. Actin polymerisation is an ATP-dependent process. Moreover, this molecule is the most important molecule of sperm energy metabolism. In the sperm cells, the energy production depends on glycolysis and/or on mitochondrial oxidative (Storey 2008; see also Chap. 2).
- F-actin and G-actin.
- Levels of intracellular calcium. Intracellular calcium is a key second messenger that is involved in sperm capacitation, amongst other processes.
- Protein synthesis. In the sperm cells when the mitochondrial-type ribosomes, which translate nuclear-encoded proteins, are blocked, actin polymerisation is inhibited (Gur and Breitbart 2006).
- The fusion of outer acrosome and plasma membranes (see also Sect. 7.7.3 and Chap. 8).

From the different nodes, actin polymerisation is the most linked node and interacts with three main nodes of sperm capacitation: phospholipase D, ATP and intracellular calcium levels. For this reason, Bernabò et al. (2011) have suggested that since the actin polymerisation node is the most linked node, it could reach all the subcellular compartments affecting the whole signal transduction system of spermatozoa. This hypothesis is backed by the effects of removing the actin polymerisation node in the in silico model. In this case, some nodes involved in sperm capacitation, such as membrane fusion, F- and G-actin and mitochondrial protein translation, are excluded from the capacitation network, while others are unaltered. Thus, blocking actin polymerisation during sperm capacitation would impede the fusion of plasma and outer acrosome membranes, thereby leading the spermatozoa unable to undergo acrosome reaction.

All these events predicted by the computational model were confirmed by the same authors, by inhibiting, under capacitating conditions, actin polymerisation in spermatozoa with cytochalasin D (Bernabò et al. 2011). This inhibition led the spermatozoa unable to undergo the acrosome reaction, in the presence of solubilised zona pellucida, which is well-known for inducing acrosome reaction, as described in Chap. 8.

In contrast, the presence of cytochalasin D did not inhibit other capacitation nodes. Thus, typical membrane remodelling which then enables the fusion of plasma and outer acrosome membranes, was not affected by the ablation of actin polymerisation in the presence of cytochalasin D. Furthermore, the inhibition of

actin polymerisation did not affect either the characteristic protein phosphorylation pattern of boar capacitated spermatozoa or the translocation of the phospholipase C-γ1 (PLC-γ1). Phospholipase C-γ1 is involved in coupling actin cytoskeleton and membrane dynamics with the calcium metabolism. Thus, upon activation, PLC-γ1 migrates from the cytosol to the plasma membrane (Spungin and Breitbart 1996), where it hydrolyses PIP2 (phosphatidylinositol 4,5-bisphosphate; PI(4,5)P2) to IP3 (inositol 1,4,5-triphosphate), a calcium mobilising second messenger, and DAG (diacylglycerol), which in turn activates the protein kinase C.

Finally, the inhibition of actin polymerisation did not affect calcium metabolism either. However, intracellular calcium plays a key role in sperm capacitation, as has been widely discussed in this Chapter. Indeed, we remember here that intracellular calcium participates in the capacitation-signalling cascade, translating the extracellular stimulation to activation of other molecular systems, such as PKA and protein kinase C pathways. When sperm cells are completely capacitated, they can respond with a high increase in intracellular calcium to the interaction with the zona pellucida of oocyte, thereby triggering acrosome reaction (see also Chap. 8).

Remodelling of plasma membrane permits its fusion with outer acrosomal membrane (Breitbart et al. 2005; see also Sects. 7.6 and 7.7), but the actin network impedes the fusion of both membranes. When sperm capacitation has been completed, and acrosome exocytosis has taken place induced by ZP-proteins, an induced high increase in intracellular calcium depolymerises the actin network, thereby allowing the fusion of plasma membrane with outer acrosome membrane. However, the main concern of the interesting study conducted by Bernabò et al. (2011) is that they did not observe an increase in acrosome-reacted spermatozoa when incubating the sperm cells with cytochalasin D. This should have been expected if the role of the actin network in separately maintaining both membranes had been crucial. In the future, further research should address this concern and other aspects that remain to be elucidated.

To date, and from the literature currently available, we can suggest that actin polymerisation appears to play a major coordination role in all the cellular districts involved in capacitation (Bernabò et al. 2010b, 2011). This hypothesis is in agreement with the newly emerging evidence that in different cellular systems the cytoskeleton not only plays a mechanical support function, but it also participates in cell signalling, especially if one considers the surface of cytoskeleton filaments on which proteins and other cytoplasmic components can dock (Janmey 1998). It is thus possible to hypothesise that another level of cell function regulation, through the diffusion along cytoskeletal networks, exists, this being an alternative to the other known route of intracellular signal transduction, such as intracellular calcium or ATP (Shafrir et al. 2000; Forgacs et al. 2004).

In short, spermatozoa behave as complex systems, also in terms of their capacitation and their ability to bind and fuse with the oocyte. Thus, spermatozoa acquire their fertilising ability when they are considered in their entire signalling network, in such a way that if a single element of the network is unperturbed by an external factor, but the coordination among all elements is broken, the spermatozoon is unable to respond to ZP-proteins.

7.13 Conclusions

Around ovulation, spermatozoa are released from the oviductal epithelium and become free within the oviductal fluid. Then, spermatozoa continue capacitation and, at an early step of this process, AQN-1 is shed from the surface. This allows the unmasking of three proteins (AWN, AQN-3 and P47/SED1) that are also attached to the sperm surface and mediate the interaction with zona pellucida. During capacitation, sperm cells undergo different changes that affect all the subcellular compartments, and can be divided into early/fast and late/slow events. Within these changes, we can find the increase in certain intracellular messengers, such as Ca^{2+} and cAMP, and the reorganisation of proteins and lipids of sperm plasmalemma. Finally, the sperm plasma and the outer acrosome membranes become more unstable and gradually acquire the ability to fuse with each other. Fusion between these two membranes will only take place when the spermatozoon interacts with the zona pellucida of the oocyte, as we will see in the next chapter.

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