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# Review and Hypothesis Factors Regulating Sperm Capacitation

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Abbreviations: ZP: zona pellucida; ICSI: intracytoplasmic sperm injection; PKA: protein kinase A; PTK: protein tyrosine kinase; BSPs: bovine seminal plasma proteins; PDE: phosphodiesterase; sAC: soluble adenylyl cyclase; VDCC: voltage-dependent calcium channel; SERCA: sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; AKAPs: A-kinase anchoring proteins; CABYR: calcium-binding tyrosine phosphorylation-regulated protein; ACRBP: acrosin binding protein; VCP: valosin-containing protein; SFK: Src-family tyrosine kinase; FGFR: fibroblast growth factor receptor; MAPK: mitogenactivated protein kinase; ERK: extracellular signal-regulated kinase.

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Capacitation is broadly defined as the functional modifications rendering sperm competent to fertilize, encompassing the ability of the sperm to bind the zona pellucida and subsequently undergo the acrosome reaction, hyperactivated motility, and the capacity to fuse with the oocyte. Although discovered in 1951, research over the past 15 years has considerably clarified the mechanisms leading to capacitation. The purpose of this review is to discuss the challenges of studying capacitation and to summarize recent notions regarding its regulation. Of particular interest is an atypical soluble adenylyl cyclase that is stimulated by bicarbonate to activate protein kinase A and drive sperm protein tyrosine phosphorylation. The identities of the phosphorylated sperm-protein substrates and the kinase(s) responsible for their tyrosine phosphorylation have fostered major questions regarding this pathway. Recent investigations, however, have made exciting advances toward resolving these queries. Advanced proteomic approaches have revealed the tyrosine phosphorylated substrates to be implicated in a diverse range of cellular activities. SRC tyrosine kinase is a particularly interesting candidate as the mediator of the protein kinase A-driven sperm protein tyrosine phosphorylation. Future studies are merited to fully characterize additional signaling mediators such as phosphatases and other kinases that may be involved, to elucidate the functional importance of the tyrosine phosphorylation on those particular substrates and to appreciate the differences that may exist among species.

**KEYWORDS** bicarbonate, calcium, cholesterol, protein tyrosine phosphorylation

### INTRODUCTION: DEFINITIONS AND ENDPOINTS

In 1951, Austin and Chang independently discovered that mammalian sperm must reside in the female reproductive tract for a period of time to attain fertilizing capacity [Austin 1951; Chang 1951]. Shortly thereafter, this interval between sperm deposition in the female at mating and the time during which fertilization occurs was termed 'capacitation' [Austin 1952].

Presently, capacitation is more broadly defined as the functional modifications that render sperm competent to fertilize an oocyte. *In vivo*, both the male and female reproductive tracts influence these modifications. Capacitation can now be reproduced *in vitro* in defined conditions for many species, with varying degrees of success for each species. It is generally accepted that the functional changes due to capacitation include: (i) the ability of the sperm to bind the oocyte's extracellular matrix, the zona pellucida (ZP; [Saling et al. 1978; Si and Olds-Clarke 1999; Topper et al. 1999]) and subsequently undergo the acrosome reaction [Saling et al. 1979; Florman and First 1988], (ii) hyperactivation, the whiplash flagellar motion required to penetrate the egg [Ho and Suarez 2001], and (iii) the capacity to fuse with the oocyte [Evans and Florman 2002]. Hyperactivation, however, is regulated by similar yet distinct signaling events that distinguish it from capacitation [Marquez and Suarez 2004].

A major hallmark of capacitation is the ability of the sperm to undergo the acrosome reaction in response to the oocyte's ZP [Saling et al. 1979; Florman and Storey 1982]. The acrosome reaction is a regulated, calcium-dependent, exocytotic event triggered by sperm binding to the ZP, in which the outer membrane of the acrosome fuses at multiple points with the overlying plasma membrane [Barros et al. 1967], liberating the acrosomal contents [Franklin et al. 1970] and exposing the molecules present on the inner acrosomal membrane. Essential to fertilization, only acrosome-reacted sperm are able to pass through the ZP and fuse with the plasma membrane of the oocyte [Yanagimachi 1994]. The resulting acrosome reaction occurs only if the sperm has previously undergone capacitation, therefore, both phenomena are obligate for fertilization, perhaps with the exception of ICSI (intracytoplasmic sperm injection), which bypasses gamete binding and fusion by artificially inserting the sperm into the metaphase II oocyte.

## EVALUATING CAPACITATION

Elucidating the molecular basis of capacitation has proved to be challenging, largely because it is difficult to establish which sperm modifications are necessary for fertilizing ability. An obvious difficulty is that capacitation is not accompanied by morphological changes. Indirect assays of capacitation are

thus used, such as the ability to undergo the acrosome reaction, which can be induced by various compounds such as calcium ionophores, homologous isolated ZP, or progesterone. Other indirect capacitation assays include chlortetracycline staining [Ward and Storey 1984], sperm agglutination [Harayama et al. 1999], and acrosin conversion [Moos et al. 1991]. Another factor obscuring the mechanism of capacitation is that it is a time-dependent process that depends on the lipid composition of the plasma membrane [Davis 1981]. Combined with the sheer number of sperm and the heterogeneity of the sperm population, it is difficult to accurately assess the temporal sequence of physiological events that occur during capacitation. Indeed, a heterogeneous sperm population widens the window of capacitation thereby prolonging the fertilizing potential of the ejaculate *in vivo* [Harrison 1996]. This is beneficial for internal fertilization as the sperm must await the ovulated oocyte. Finally, most studies on capacitation occur *in vitro* and extrapolating such results to the *in vivo* condition might not be fully accurate [Kopf et al. 1999; Suarez and Pacey 2006]. As a consequence, decisively determining genuine capacitation is complicated.

## CAPACITATION AS A REGULATED EVENT

Despite the technical challenges of assaying capacitation, the consensus is that it is a regulated, signal transduction-mediated event. Unlike most other signaling cascades, however, capacitation is particular in that it appears to occur without a specific ligand binding to a precise sperm receptor [Storey 1995], and can occur spontaneously in defined media *in vitro*. That is, no primary molecular binding event appears to be necessary to trigger the intracellular pathways leading to sperm fertilizing ability, which is in stark contrast to classical examples of signal transduction-mediated reactions such as hormone-receptor binding, T-cell antigen binding, or nervous system signaling (e.g., odorant receptor and adrenergic receptor activation).

That capacitation can occur spontaneously *in vitro* in defined media without the inclusion of biological materials suggests it is intrinsically regulated by mechanisms involving pre-programmed reactions [Kopf et al. 1999]. Therefore, once it is triggered by

some external event, capacitation progresses essentially following a pre-programmed pathway. This feature is well-illustrated by 'cryo-capacitation' or functional capacitation induced by freezing sperm; after thawing, a proportion of the sperm are able to fertilize even in the absence of traditional capacitation stimulators *in vitro* [Cormier et al. 1997; Bailey et al. 2003]. Capacitation is controlled by both extrinsic and intrinsic factors with the major extrinsic signals mediating capacitation being sterol acceptors (traditionally albumin), bicarbonate, and calcium. Together, these signaling molecules activate the protein kinase A (PKA) and protein tyrosine kinase (PTK) pathways that drive capacitation [Visconti et al. 1995a; 1995b].

## **CAPACITATION INITIATES AT THE PLASMA MEMBRANE**

### **Decapacitation Factors and Sterol Acceptors**

The plasma membrane is the barrier between the cell's external and internal environments, which represents the starting point of capacitation. Early research demonstrated that capacitation is associated with alterations to sperm surface components (reviewed by [Yanagimachi 1994]). 'Decapacitation factor' is a general term for molecules present in seminal plasma that coat and stabilize the sperm surface, likely to prevent premature capacitation, which then are removed during incubation to promote capacitation at a later, more appropriate time. Among the best characterized decapacitation factors are the bovine seminal plasma proteins (BSPs; now known as the Binder of Sperm family), which bind to sperm choline phospholipids, then are stripped from sperm by heparin or components in the genital tract of the cow, taking certain membrane lipids (cholesterol and phospholipids) along with them [Thérien et al. 1998]. A particular BSP, PDC-109 (also known as BSP-A1/A2) is responsible for binding of bull sperm to the sperm storage reservoir within the oviductal epithelium [Gwathmey et al. 2006]. Interestingly, bovine sperm that are already capacitated before being added to oviductal explants, have a reduced binding affinity for the oviduct epithelium/sperm reservoir [Lefebvre and Suarez 1996]. These observations suggest that

PDC-109 from the seminal plasma coats sperm during ejaculation and enables binding to the oviductal sperm reservoir. Binding to oviductal cells prolongs sperm viability and retards capacitation [Boilard et al. 2002]. Although BSPs and their homologues bind sperm, their binding alone does not lead to capacitation. The presence of PDC-109 in cholesterol-poor membranes, however, disrupts membrane structure [Tannert et al. 2007]. A putative mechanism by which sperm undergo capacitation in the oviduct could involve binding of heparin-like molecules present in the oviductal fluid to PDC-109, thereby displacing it from the epithelium and releasing the sperm from the reservoir. The PDC-109 would subsequently be removed along with membrane cholesterol, triggering the signaling cascade leading to capacitation.

For successful capacitation *in vitro*, serum albumin has traditionally been included in the medium [Visconti et al. 2002], and is thought to serve as a sink for the removal of cholesterol from the sperm plasma membrane [Go and Wolf 1985; Langlais et al. 1988; Cross 1998]. During epididymal maturation, large amounts of cholesterol are incorporated into the sperm, presumably to stabilize the plasma membrane during transit in the female tract (discussed by [Yanagimachi 1994]). Supporting the hypothesis that cholesterol removal is required for capacitation are observations that other non-albumin cholesterol acceptors can replace albumin, such as high density lipoproteins (bovine; [Thérien et al. 1998]; murine; [Visconti et al. 1999]) and beta-cyclodextrins (murine; [Choi and Toyoda 1998]; human; [Parinaud et al. 2000]; bovine; [Nagao et al. 2010]). Supplementing the medium with exogenous cholesterol sulphate or desmosterol blocks the action of albumin and prevents capacitation [Cross 1998; Visconti et al. 1999]. Moreover, glycodeclin-S, a major glycoprotein in human seminal plasma, has a 'decapacitating' effect on sperm by reducing albumin- and cyclodextrin-induced cholesterol effluxes, and suppressing capacitation [Chiu et al. 2005].

It is clear that sperm are protected against premature capacitation in the epididymis, that seminal plasma contains stimulators and inhibitors of capacitation, and that the female tract can both prevent and encourage capacitation, likely to ensure the availability of a sufficient number of functional sperm once the ovulated oocyte(s) reaches the ampulla.

## Importance of Reduced Membrane Cholesterol and Rafts

Davis [1981] proposed that the cholesterol:phospholipid ratio is a major determinant of capacitation, and removal of cholesterol decreases this ratio to destabilize the sperm head plasma membrane, thus increasing its potential for fusion with the outer acrosomal membrane. Cholesterol removal alters the protein-lipid organization of the sperm head plasma membrane [Langlais et al. 1988; Lin and Kan 1996] and increases its fluidity [Wolf et al. 1986; Smith et al. 1998]. By blocking cholesterol loss, Cross [1998] also prevented the sperms' capacity to respond to the calcium ionophore. Although these sperm display increased internal calcium, they do not acrosome react, indicating that the removal of cholesterol does not simply facilitate calcium influx. Rather, a downstream event in the signal transduction pathway seems impeded by a high membrane cholesterol:phospholipid ratio.

It is now understood that the lipid organization within biological membranes, including sperm, is heterogeneous and includes rafts. Rafts are defined as dynamic sub-compartments of the membrane bi-layer, enriched in cholesterol and sphingolipids that form membrane signaling and trafficking platforms [Lingwood and Simons 2010]. Selvaraj et al. [2009] recently reported the presence of sterol-rich microdomains in the membrane overlying the acrosome in live, unfixed murine and human sperm. Using methyl-beta-cyclodextrin, Shadan et al. [2004] showed that capacitation-associated cholesterol efflux did not alter the composition of the lipid rafts in boar sperm, which was rather unexpected. They suggested that a targeted loss of cholesterol from the non-raft pool promotes raft migration over the anterior sperm head. Similarly, in boar sperm, others have reported dissociation of lipid rafts during capacitation [Sleight et al. 2005] and the acrosome reaction [Miranda et al. 2009]. It has been suggested that during capacitation, lipid rafts with enhanced ZP-binding potential cluster to reposition sperm membrane proteins that are involved in gamete recognition, or form signaling complexes leading to downstream reactions later, during capacitation or acrosomal exocytosis [van Gestel et al. 2005; Bou Khalil et al. 2006]. Supporting this hypothesis, isolated detergent-resistant membrane fractions from mouse sperm selectively bound homologous unfertilized

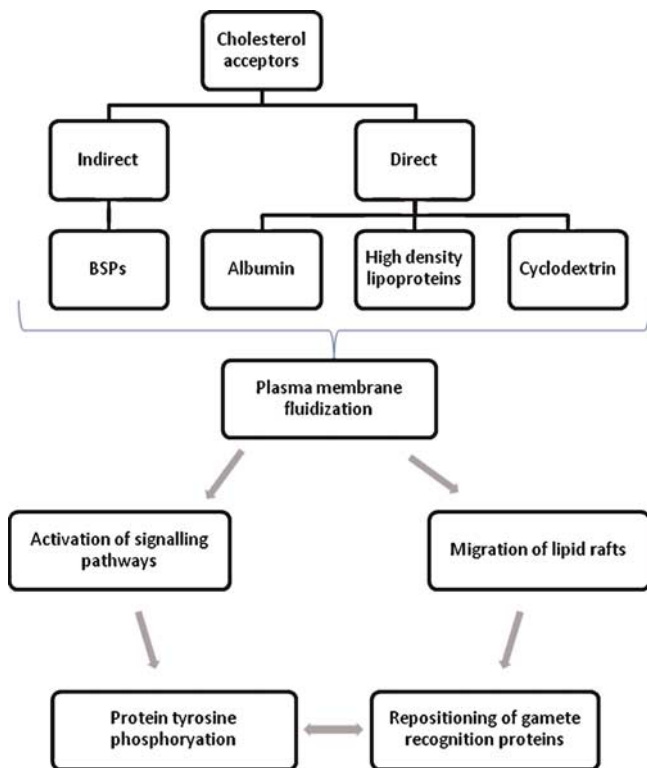
oocytes, compared to fertilized oocytes [Nixon et al. 2009]. Proteomic analysis of these rafts revealed 100 proteins, many of which have been implicated in gamete interactions.

Membrane fluidization induced by cholesterol removal is known to activate a signal transduction pathway leading to the tyrosine phosphorylation of sperm proteins [Visconti et al. 1999; Shadan et al. 2004]. Cholesterol removal also promotes the binding of Rab3A (involved in the membrane fusion/secretory pathway) to the sperm plasma membrane [Belmonte et al. 2005]; cholesterol thus indirectly regulates the acrosome reaction. In uncapacitated boar sperm, the fusion SNARE molecules, syntaxins and VAMP, are scattered over the entire head, but following cholesterol depletion, relocalize to the apical ridge, which is the site of sperm zona binding leading to the acrosome reaction [Tsai et al. 2007]. Redistribution to and from rafts could be facilitated by the architectural structure of the microdomain itself, within the bilayer [Shadan et al. 2004; Sleight et al. 2005; Van Gestel et al. 2005; Bou Khalil et al. 2006], as well as via scaffolding proteins. The multi-PDZ domain protein, MUPP1, is present in detergent-resistant fractions of both noncapacitated and capacitated mouse sperm, and it is involved in tethering and docking of the acrosome, and subsequent activation of a SNARE fusion mediator, syntaxin2 [Ackermann et al. 2008].

Therefore, many membrane modifications tend to be architectural, which influence the behavior of the signaling molecules associated with the membrane or underlying cytoskeleton, such as ion channels, pumps, or enzymes. Figure 1 summarizes the process of plasma membrane fluidization.

## THE MANY ROLES OF BICARBONATE

Fluids of the female reproductive tract are rich in bicarbonate [Rodriguez-Martinez et al. 2005; Zhou et al. 2005], which likely influences sperm capacitation, although the sperm reservoir secretions are apparently bicarbonate-poor [Rodriguez-Martinez et al. 2005]. Bicarbonate is particularly responsible for inducing capacitation as it rapidly increases the fluidity of boar sperm membranes [Harrison et al. 1993]. Indeed, pig sperm can undergo capacitation *in vitro* in BSA-free conditions; however, bicarbonate is essential [Tardif et al. 2003]. It appears that there is a certain synergy between the destabilizing effects of bicarbonate and



**FIGURE 1** The fluidization of sperm head plasma membrane is a critical, early step of capacitation. Sterol removal is the principal means by which membrane fluidization occurs and can be mediated *in vitro*, indirectly or directly via various acceptor molecules. Increased membrane fluidity repositions lipid rafts, which contain proteins involved in gamete recognition, thereby facilitating sperm-egg interactions and membrane fusion. Membrane fluidization also appears to be required for the onset of signaling events that characterize capacitation.

BSA on the bilayer, as bicarbonate fluidized boar sperm membranes, permitting calcium influx to the head and tail that was enhanced by albumin [Harrison et al. 1993]. Similarly, human sperm seem to require both bicarbonate and BSA for protein phosphorylation, which is reversible upon incubation in bicarbonate/BSA-free medium. However, removal of only one of these two components reverses the cytosolic calcium increase and acrosomal responsiveness [Bedu-Addo et al. 2005].

### Bicarbonate, Sperm Membrane Structure, and Function

Gadella and Harrison [2000] demonstrated numerous compositional changes in phospholipid distribution across boar sperm membrane bilayers, due to bicarbonate. Specifically, bicarbonate activated the outward translocation of sphingomyelin, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine,

possibly by activating a ‘scramblase’ activity. In addition, these authors noted phospholipase activation and membrane fluidization due to bicarbonate. The effect of the bicarbonate-induced scramblase activity could vary among different regions of the sperm according to the specific lipid population, thus reducing phospholipid asymmetry over the head, but not in the flagellum [Gadella and van Gestel 2004]. Such observations are relevant to capacitation because both decreased phospholipid asymmetry and phosphatidylserine exposure facilitate cell-cell adhesion, which would enhance sperm-oocyte interaction [Schlegel et al. 1985; Closse et al. 1999; Gadella and Harrison 2000]. Wang et al. [2004] described a sperm-specific, flippase-like aminophospholipid transporter in the acrosomal region that helps establish membrane phospholipid asymmetry; null mice showed phospholipid scrambling before a bicarbonate challenge. This premature membrane fluidization prior to capacitation compromised male fertility, demonstrating the importance of proper phospholipid asymmetry and its regulated collapse [Wang et al. 2004]. It is noteworthy that bicarbonate is required for migration of protein DE (CRISP-1) to the equatorial segment, and oocyte fusion ability in rat sperm [Da Ros et al. 2004].

Roldan and Vazquez [1996] showed that bicarbonate/ $\text{CO}_2$  activated phospholipase  $\text{A}_2$  was present in pig sperm and enhanced their ability to acrosome react, presumably via the release of arachadonic acid, which has long been known to promote the acrosome reaction. Arachadonic acid is fusogenic and activates membrane fusion proteins [Darios et al. 2007], but can also regulate voltage-gated calcium channels [Roberts-Crowley et al. 2009]; these functions, however, have yet to be demonstrated in sperm.

### Bicarbonate Stimulates a Soluble Adenylyl Cyclase

Bicarbonate induces these changes to membrane architecture via a cAMP-dependent protein kinase (Protein Kinase A, PKA) pathway, because the inclusion of cAMP analogues, cyclic nucleotide phosphodiesterase (PDE) inhibitors (which prevent cAMP degradation), and phosphatase inhibitors (which prolong PKA-mediated protein phosphorylation) all mimicked the effect of bicarbonate [Gadella and Harrison 2000]. Harrison and Rapid [2004] also

showed that bicarbonate catalyses PKA-dependent phosphorylation of sperm proteins.

It is now accepted that a major regulatory role of bicarbonate on capacitation is mediated by a non-transmembrane soluble adenylyl cyclase (sAC), which is distinct from classical G protein-regulated, transmembrane adenylyl cyclases. Bicarbonate can directly activate the sAC in a pH-dependent manner both *in vivo* and *in vitro*, with the EC50 of this stimulation being ~25 mM, equivalent to the bicarbonate concentration in serum and capacitation media [Chen et al. 2000]. Mice lacking sAC have morphologically normal testes, epididymides, and sperm, but are infertile due to major sperm motility defects [Esposito et al. 2004]. Exogenous cAMP analogues only partly rescued the motility parameters, the sperm remained infertile *in vitro*, and the sAC deficient mice were subsequently determined to have functional defects beyond motility [Hess et al. 2005]. Using a sAC inhibitor that completely abolishes bicarbonate-induced sperm cAMP production, Hess et al. [2005] demonstrated that sAC during capacitation is required for sperm protein tyrosine phosphorylation and fertility, but not for hyperactivation or the acrosome reaction, both of which are largely regulated by classical membrane-bound adenylyl cyclases in a bicarbonate-independent manner.

### **Bicarbonate Increases pH<sub>i</sub> and Induces Membrane Hyperpolarization**

Bicarbonate is the major buffering ion in sperm, and capacitation is accompanied by intracellular alkalinization [Parrish et al. 1989]. Increasing pH<sub>i</sub> in bovine sperm by incubating in 1% CO<sub>2</sub> in air is sufficient to induce protein phosphorylation and acrosomal responsiveness, suggesting that alkalinization drives sAC activity [Galantino-Homer et al. 2004]. The stimulation of sAC activity in sperm by bicarbonate increases cAMP [Garty and Salomon 1987; Visconti et al. 1990], which is a central regulator of capacitation.

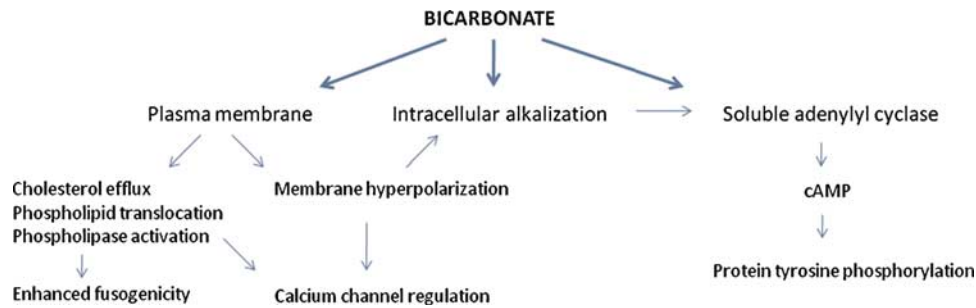
Capacitation is also accompanied by hyperpolarization of the plasma membrane in mouse sperm, apparently due to enhanced K<sup>+</sup> permeability [Zeng et al. 1995; Martínez-López et al. 2009]. Also in mouse sperm, BSA (or beta-cyclodextrin) and

bicarbonate both are necessary for this membrane hyperpolarization, which leads to intracellular alkalinization and protein tyrosine phosphorylation, a signature of capacitation [Demarco et al. 2003]. Mouse sperm potassium currents generated by SLO3 channels were shown to be regulated by intracellular alkalinization and cAMP, highlighting the importance of bicarbonate-mediated events on capacitation [Martínez-López et al. 2009]. Membrane hyperpolarization regulates the ability of the sperm to later generate transient calcium elevations during the acrosome reaction, and deletion of the sperm-specific, pH-dependent SLO3 potassium channel resulted membrane depolarization and infertility due to the inability of the sperm to undergo the acrosome reaction, even in the presence of calcium ionophore [Santi et al. 2010]. Together, these results suggest that cholesterol efflux and intracellular pH play a role in capacitation-induced hyperpolarization and subsequent signaling events. Although the exact role of bicarbonate on membrane hyperpolarization is unknown, Demarco et al. [2003] hypothesized that sAC and Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> co-transporters co-localize in sperm and ultimately regulate the signaling cascade leading to capacitation.

In sum, bicarbonate is a critical signaling molecule during capacitation and acts through sAC. It is also involved in PKA-mediated lipid bilayer redistribution and subsequent membrane dynamics (an attractive hypothesis is that sAC activates a 'scramblase', which is potentiated by cholesterol removal), as well as membrane hyperpolarization necessary to complete capacitation. Figure 2 briefly summarizes some of the principal roles of bicarbonate during capacitation.

### **CALCIUM AND CAPACITATION Increased Intracellular Calcium during Capacitation**

Separating the effect of calcium during capacitation from its role during the acrosome reaction is challenging; different mechanisms of calcium entry into sperm seem to regulate these two phenomena [de Lamarinde et al. 1997]. Studies of various species have indicated that capacitation is calcium-dependent (e.g., bovine; [Handrow et al. 1989]; mouse; [Visconti et al. 1995a]; porcine; [Tardif et al. 2003]), and



**FIGURE 2** Summary of the putative roles of bicarbonate during sperm capacitation. Bicarbonate is thought to act on the plasma membrane to influence membrane architecture and intracellular pH. Also, bicarbonate directly stimulates soluble adenylyl cyclase. Functionally, bicarbonate prepares the sperm for acrosomal exocytosis: ion currents are altered and signaling pathways are initiated, ultimately facilitating membrane fusion.

accompanied by elevated cytosolic calcium [Dubé et al. 2003], specifically in the head/acrosome [Parrish et al. 1999; Feng et al. 2006]. Increased intraflagellar calcium occurs during capacitation [Suarez et al. 1993], which is more involved in hyperactivated motility than in capacitation per se; however, the mechanism by which calcium enters is confounded with capacitation itself.

Capacitation-associated alkalinization participates in increasing intracellular calcium levels during late capacitation/hyperactivation, facilitating the acrosome reaction [Santi et al. 2010]. In human sperm, voltage-dependent calcium channel (VDCC) activity is highly sensitive to the increased pH<sub>i</sub> during capacitation so that a combined effect of alkalinization and biochemical regulation enhances calcium influx through these channels [Neri-Vidaurri Pdel et al. 2006]. As well, the constitutively active CATSPER family of sperm-specific flagellar calcium channels are strongly potentiated by intracellular alkalinization to increase intracellular calcium and induce hyperactivation in mammalian sperm [Quill et al. 2001; Kirichok et al. 2006; Jin et al. 2007]. Xia and Ren [2009] recently used CATSPER1 null mice to show that during capacitation, the increased intracellular calcium induced by BSA requires CATSPER, but not the VDCC. These findings are a step towards identifying the molecular conduit responsible for the calcium entry required for the capacitation-associated hyperactivated motility. Coupled with the observation in mice that a pH-dependent potassium channel (SLO3) is essential for membrane hyperpolarization during capacitation and calcium responsiveness leading to the acrosome reaction [Santi et al. 2010], it is clear that motility and gamete interactions are driven by related, but distinct, pathways.

## Calmodulin, Ca<sup>2+</sup>-ATPase Activities and Capacitation

Several studies have shown that in the presence of heparin, which is necessary for *in vitro* capacitation of bovine sperm, there is reduced expression of calmodulin binding proteins and calmodulin content, and consequently, less calmodulin binding to sperm proteins [Leclerc et al. 1989; 1990; 1992; Leclerc and Goupil 2000]. Plasma membrane Ca<sup>2+</sup>-ATPase is regulated by calmodulin binding and the capacitation-associated calcium influx might be due to reduced calmodulin-mediated Ca<sup>2+</sup>-ATPase activity [Leclerc and Goupil 2000; Feng et al. 2006]. Indeed, calmodulin in the bovine sperm head [Leclerc and Goupil 2000], and plasma membranes from mouse sperm heads have Ca<sup>2+</sup>-ATPase activity that is stimulated by calmodulin as well as by some decapacitation factor [Adeoya-Osiguwa and Fraser 1996]. Furthermore, treating bovine sperm with an inhibitor of calcium-calmodulin dependent PDE type 1 (PDE1) mimicked the effect of heparin on bovine sperm, suggesting that decreased calmodulin during capacitation would reduce PDE1 activity, thereby increasing cAMP [Fournier et al. 2003]. Therefore, calmodulin, a calcium-binding protein considered to be a major transducer of calcium signals, seems to be reduced during capacitation, which would increase sperm calcium levels, perhaps by inhibiting plasma membrane Ca<sup>2+</sup>-ATPase, and enhance cAMP by inhibiting PDE1.

In addition to plasma membrane Ca<sup>2+</sup>-ATPases, intracellular sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA)-like calcium pumps are involved in filling internal calcium stores, because the SERCA inhibitor, thapsigargin, increases sperm cytosolic calcium in bovine [Parrish et al. 1999] and human

sperm and also provokes protein phosphorylation typical of capacitation [Dorval et al. 2002]. It was proposed that during capacitation, the initial influx of calcium into sperm is used to fill an intracellular calcium store located in the acrosome [Parrish et al. 1999] and that sperm SERCAs are inhibited later during capacitation, permitting the rise in cytosolic calcium and subsequent appearance of tyrosine phosphoproteins [Dorval et al. 2002; Feng et al. 2006]. SERCA 2 has indeed been identified in the outer acrosomal membranes and midpiece of human, bovine, and mouse sperm [Lawson et al. 2007]. Furthermore, thapsigargin treatment of capacitated sperm will induce the acrosome reaction [Parrish et al. 1999; Dorval et al. 2003], underscoring the challenge of dissociating the role of calcium as a signaling molecule during capacitation, compared to during the acrosome reaction. In this sense, it is interesting that Kim and Gerton [2003] reported that increased intracellular calcium causes sperm to cross a 'capacitation threshold'; a further rise causes sperm to cross an 'exocytosis threshold'. This observation helps explain spontaneous versus induced acrosome reactions, and is a direct consequence of calcium entry into the sperm during capacitation.

## **PROTEIN PHOSPHORYLATION AND KINASES INVOLVED IN CAPACITATION**

Phosphorylation is a post-translational modification of proteins that is important in regulating numerous cellular activities, including sperm function (reviewed by [Urner and Sakkas 2003] and [Naz and Rajesh 2004]). Since mature sperm are transcriptionally quiescent, protein phosphorylation is an invaluable method to control their processes. Protein kinases, which phosphorylate specific targets to modulate their function, and protein phosphatases, which dephosphorylate the molecules, control protein phosphorylation.

### **Protein Kinase A and Tyrosine Phosphorylation of Sperm Proteins**

It has been known for some time that cyclic nucleotides play key roles during sperm capacitation, based on observations that PDE inhibitors and/or cAMP analogues accelerate capacitation and *in vitro* fertilization (reviewed by [Fraser and Monks

1990]). Direct evidence for the role of cAMP was shown in bovine sperm, as capacitation induced by either heparin or oviductal fluid involves an increase in cAMP [Parrish et al. 1994]. In a related study, treatment with a cAMP antagonist did not prevent the increase in pHi associated with heparin-induced capacitation [Uguz et al. 1994], suggesting that cAMP activation and presumable protein phosphorylation events are either independent of, or occur downstream of the pHi rise. Indeed, protein phosphorylation in bovine sperm can be induced by cytosolic alkalinization, suggesting that the bicarbonate-mediated pHi rise is coupled to sAC activation and drives the cAMP/PKA pathway of capacitation [Galantino-Homer et al. 2004]. As outlined earlier, the bicarbonate-mediated changes in sperm bilayer architecture are mediated by PKA [Gadella and Harrison 2000; Harrison and Rapid 2004] and an increase in cAMP is indeed detected very early (within minutes) during incubation of boar sperm in capacitating conditions [Tardif et al. 2004].

Naz et al. [1991] reported that increased PTK activity and tyrosine phosphorylation in human sperm extracts are associated with capacitation. In a landmark report, Visconti et al. [1995a] showed that the tyrosine phosphorylation of a subset of proteins occurs during capacitation of mouse epididymal sperm, and that albumin, bicarbonate, and calcium are all necessary. Moreover, they also demonstrated that cAMP analogues bypassed the requirement of these three components, and concluded that PKA-dependent protein tyrosine phosphorylation leads to capacitation in mouse sperm [Visconti et al. 1995b]. A similar pathway, which is unique to sperm, has been subsequently reported for numerous species, including human [Leclerc et al. 1996], bovine [Galantino-Homer et al. 1997], cat [Pukazhenthil et al. 1998], and horse [Pommer et al. 2003] sperm, among others. In mouse sperm, this tyrosine phosphorylation depends on membrane cholesterol removal [Visconti et al. 1999] and, as mentioned earlier, in bovine sperm, tyrosine phosphorylation follows the heparin-induced pHi rise [Galantino-Homer et al. 2004]. Finally, PKA $\alpha$ II knockout mice are infertile due to the absence of increased sperm protein tyrosine phosphorylation under capacitating conditions, which is consistent with a central role for PKA in regulating this cascade [Nolan et al. 2004].

## Identification and Importance of the Tyrosine Phosphoproteins

The pertinence of this protein tyrosine phosphorylation to capacitation is not fully established. In the mouse, the appearance of a cohort of tyrosine phosphorylated proteins is accepted as essential for acrosomal responsiveness and fertilization *in vitro* [Visconti et al. 1995a; 1995b], and is thus considered as an endpoint of capacitation. Others suggest that tyrosine phosphoprotein appearance is not always an endpoint of capacitation (porcine; [Tardif et al. 2003]). However, fertilization in the absence of tyrosine phosphorylation has not been reported.

The development of proteomic techniques has led to the identification of a number of capacitation-associated tyrosine phosphoproteins, providing a good starting point for hypothesis-driven study [Ficarro et al. 2003; Baker et al. 2010; Nixon et al. 2010]. Early studies identifying capacitation-induced, tyrosine phosphorylated sperm proteins revealed many to be localized to the flagellum, suggesting importance during hyperactivation. A-kinase anchoring proteins (AKAPs) sequester PKA to subcellular locations, and the most prominent tyrosine phosphoproteins appearing in capacitated human sperm are AKAP82 (now known as AKAP4) and proAKAP82, which are localized to the cytoskeletal fibrous sheath of the principal piece [Carrera et al. 1996]. These findings lent support to an interaction between PKA and PTK pathways, while suggesting that protein tyrosine phosphorylation may be part of a signal transduction pathway likely involved in hyperactivation. Similarly, CABYR (calcium-binding tyrosine phosphorylation-regulated protein) undergoes tyrosine phosphorylation during capacitation and is localized to the principal piece of the human sperm flagellum in association with the fibrous sheath [Naaby-Hansen et al. 2002]. Interestingly, CABYR gains calcium-binding capacity when tyrosine phosphorylated during capacitation, although the functional implication of the calcium binding is not known.

Some tyrosine phosphoproteins appear over the sperm head during capacitation, and these molecules are presumably more significant to the outcome of capacitation than those of the flagellum. During capacitation, boar sperm seem to experience a marked increase in the tyrosine phosphorylation of head-surface proteins [Tardif et al. 2001]. A major

tyrosine phosphoprotein in capacitated pig sperm is ACRBP (also known as sp32), a (pro)acrosin binding protein [Dubé et al. 2005]. ACRBP is present in the acrosome, which brings forth the intriguing questions of why would an acrosomal protein require phosphorylation, and which kinase is present and functional in the acrosome? ACRBP was also shown to be tyrosine phosphorylated during human sperm capacitation [Ficarro et al. 2003] and has ZP binding potential [van Gestel et al. 2005]. Moreover, two plasma membrane tyrosine phosphoproteins (35 and 46 kDa), isolated from capacitated boar sperm, have high ZP binding affinity [Flesch et al. 2001].

Asquith et al. [2004] showed that phosphotyrosine expression associated with sperm capacitation is present on the exterior head surface of live mouse sperm, and that the molecular chaperones, endoplasmic and heat shock protein 60 are two such tyrosine phosphoproteins. Sperm-oocyte binding assays revealed that almost all sperm bound to the ZP demonstrated head tyrosine phosphoprotein localization, suggesting that tyrosine phosphorylation plays a role in remodeling the sperm surface to enable ZP recognition. It was proposed that during the early stages of capacitation and the initiation of cAMP signaling, changes in plasma membrane architecture and activation of molecular chaperones by tyrosine phosphorylation induce conformational changes to expose the ZP recognition complex on the sperm head surface [Asquith et al. 2004].

Phosphoproteome analyses of human sperm revealed that at least 18 proteins are tyrosine phosphorylated during capacitation [Ficarro et al. 2003]. Valosin-containing protein (VCP), a homologue of the SNARE-interacting protein, NSF, is tyrosine phosphorylated after capacitation and relocates from the connecting piece to the anterior head [Ficarro et al. 2003]. SNARE proteins regulate membrane fusion and exocytosis; therefore, tyrosine phosphorylation of VCP could either directly mediate acrosomal responsiveness, or act as a chaperone to carry membrane fusion proteins to the site of plasma-acrosomal membrane fusion [Ficarro et al. 2003]. Indeed, tyrosine phosphorylated NSF is maintained in an inactive state and protein tyrosine phosphatase action reduces the phosphotyrosine content of NSF to promote membrane fusion [Huyh et al. 2004]. Therefore, tyrosine

phosphorylation might inactivate VCP to prevent premature acrosomal exocytosis.

In sum, this discussion represents only a brief overview of the tyrosine phosphorylated proteins identified to date. The diversity of these substrates suggests that capacitation is accompanied by motility shifts, functional changes to mitochondria, sperm surface remodeling, acrosomal changes possibly related to exocytosis, and preparing the sperm for fusion.

## WHAT ABOUT PROTEIN TYROSINE KINASES?

Despite the importance of protein tyrosine phosphorylation during capacitation, the enzymes (kinases, phosphatases) involved are not fully characterized. Protein tyrosine kinase activity was elevated in capacitated human sperm extracts [Naz et al. 1991], although the kinase itself was not further characterized and substrates were not determined.

The non-receptor PTK YES1 (c-Yes) has been identified in the head membranes of human sperm [Leclerc and Goupil 2002] and is stimulated in cAMP-elevating conditions that also increase protein phosphotyrosine content. However, YES1, which is a member of the Src family, does not have a cAMP-dependent phosphorylation site, so the mechanism by which cAMP stimulates this kinase is unclear. Moreover, it is negatively regulated by calcium, which is inconsistent with the calcium-dependency of capacitation-associated protein tyrosine phosphorylation.

However, a bioinformatics search of kinases that are known to be potentially regulated by PKA lead to the hypothesis that SRC is involved in capacitation. Indeed, the addition of SRC inhibitors prevented the appearance of tyrosine phosphoproteins in human sperm induced by wheat germ agglutinin binding to platelet cell adhesion molecule 1 (PECAM-1; [Nixon et al. 2005]). Recent studies provide convincing evidence that SRC is present in human [Lawson et al. 2008; Mitchell et al. 2008] and murine [Baker et al. 2006; Krapf et al. 2010] sperm. These authors also observed that SRC activity is enhanced by the cAMP pathway, and showed SRC association with the catalytic subunit of PKA; SRC inhibition decreased the phosphotyrosine content of sperm proteins. In human sperm, SRC is present in both the head and flagellum [Lawson et al. 2008; Mitchell et al. 2008], which would support a role for

this kinase in capacitation-related events beyond motility. In capacitated human sperm, the active tyrosine-phosphorylated SRC (pY416) was localized to the midpiece-flagellum [Mitchell et al. 2008], yet SRC kinase inhibition suppressed capacitation-associated tyrosine phosphorylation but not hyperactivation. These findings suggest that SRC is a strong candidate for mediating the PKA-dependent protein tyrosine phosphorylation that occurs during capacitation, although additional functional assays are required as confirmation.

In the mouse, SRC is predominantly located in the flagellum and functionally linked to hyperactivation induced *in vitro* by cAMP analogues [Baker et al. 2006]. In addition, Src-family tyrosine kinase (SFK) inhibitors block *in vitro* fertilization [Krapf et al. 2010], reinforcing the importance of SRC as a mediator of capacitation. Curiously, sperm from both *Src*-null and wild-type mice display similar levels of capacitation-associated tyrosine phosphorylation, suggesting either that SRC does not directly phosphorylate sperm protein tyrosine residues as part of capacitation, or that SRC deletion can be compensated for by other tyrosine kinases [Krapf et al. 2010]. Interestingly, PKA substrate phosphorylation was unaltered in sperm from the *Src*-null animals, but was blocked by SFK inhibitors. Cyclic AMP agonists did not restore phosphorylation of PKA substrates or the increase in tyrosine phosphorylation in the presence of the SFK inhibitors. That the presence of SRC is not required for sperm tyrosine phosphorylation while SFK inhibition blocked both tyrosine phosphorylation and fertilization, was unexpected. These authors propose a second, parallel pathway in which serine-threonine phosphatases are inactivated by SFK inhibition, thereby favoring PKA activity [Krapf et al. 2010]. Inactive serine-threonine phosphatases would enhance PKA phosphorylation and stimulate downstream events leading to capacitation. In light of these highly novel findings, serine-threonine phosphatase activity during capacitation warrants in-depth investigation.

Given the complexity of the subcellular localization and substrates of the capacitation-associated tyrosine phosphorylation events, it is plausible that multiple PKA-activated tyrosine kinases may participate, possibly in a species specific manner. The observation that another non-receptor tyrosine kinase, c-Abl, which is localized to the head and

flagellum of mouse sperm and whose activity is up-regulated by PKA, may be involved, is not unreasonable [Baker et al. 2009]. Inhibition of c-Abl partly decreased sperm protein tyrosine phosphorylation induced by exogenous cAMP analogues [Baker et al. 2009], giving credibility for a role in sperm capacitation, although thorough functional studies have yet to be conducted.

Various receptor PTKs have been reported in mature sperm of several species (reviewed by [Naz and Rajesh 2004]). Receptor PTK are transmembrane proteins consisting of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. Upon extracellular ligand binding, these PTK dimerize and the kinase domain undergoes tyrosine autophosphorylation. Fibroblast growth factor receptor (FGFR)-1, a receptor PTK, has recently been implicated in mouse sperm capacitation [Cotton et al. 2006]. Originally generated to study flagellar function, transgenic mice were developed to express a dominant-negative variant of FGFR-1 in male haploid germ cells. These transgenic mice revealed subfertility in part due to poor sperm production; however, those sperm that were produced had a severely compromised ability to undergo capacitation. Wild-type mice had functional FGFR-1 that suppressed downstream mitogen-activated protein kinase (MAPK) signalling and protein tyrosine phosphorylation; FGFR-1 activation thus negatively regulates capacitation [Cotton et al. 2006]. These findings indirectly support the hypothesis that the MAPK signaling cascade promotes capacitation-associated tyrosine phosphorylation.

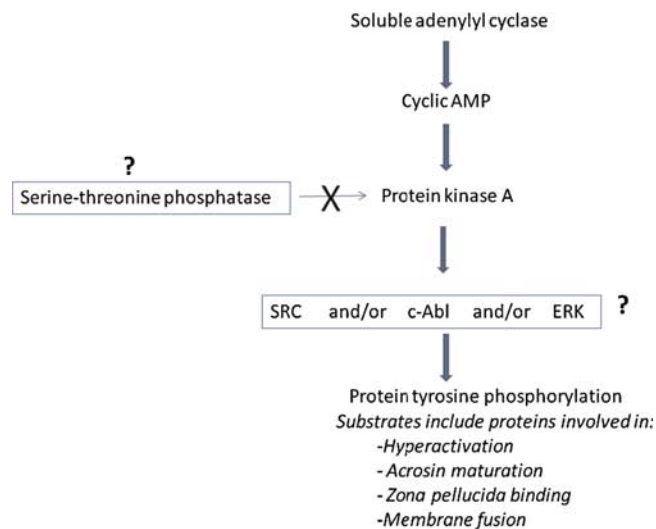
## The MAPK/ERK Pathway

Indeed, extracellular signal-regulated kinase (ERK) module of the MAPK pathway appears to play a role in generating tyrosine phosphorylated sperm proteins during capacitation, and multiple signaling cascades highlight the complexity of the capacitation process. Whereas a significant effect of PKA-mediated PTK signaling during capacitation is on sperm hyperactivation, ERK signaling seems to be particularly directed to modifications in the sperm head.

An ERK is active in human sperm during capacitation [Luconi et al. 1998] and modulation of the ERK cascade with specific inhibitors indicates that it plays a role upstream of protein tyrosine phosphory-

lation [de Lamarinde and Gagnon 2002]. Moreover, ERK1/2 activation was evident by enhanced phosphorylation during capacitation, and ERK1/2 inhibitors blocked both protein tyrosine phosphorylation and the ability of the sperm to acrosome-react. Later, O'Flaherty et al. [2005] demonstrated that typical stimulators of human sperm capacitation and protein tyrosine phosphorylation, such as fetal cord serum ultrafiltrate, BSA, cAMP analogues, and reactive oxygen species, stimulated the phosphorylation (and presumably the activation) of three MEK-like proteins. MEK is a dual-specificity kinase that phosphorylates the tyrosine and threonine residues on ERK1/2 required for activation. Interestingly, inhibition of both the PKA pathway and non-receptor PTKs blocked the MEK-like phosphorylation completely, although protein kinase C and receptor PTK inhibition only prevented the phosphorylation of one of the MEK-like proteins [O'Flaherty et al. 2005]. Together, these data indicate a complex network of differentially modulated pathways is activated for sperm capacitation.

In the mouse, Nixon et al. [2010] recently demonstrated that sperm surface phosphotyrosine expression is strongly driven by the ERK module of the MAPK pathway. Immunolabeling revealed core elements of the MAPK signaling pathway including SHC1, GRB2, RAS, RAF1, MEK, and ERK1/2, to be present primarily in the periacrosomal region. Phospho-specific antibodies revealed a dramatic increase in phosphorylation when sperm were incubated in capacitating conditions, reflecting cascade activation. Surface phosphotyrosine expression of capacitated sperm was suppressed in the presence of specific inhibitors of the ERK cascade, although levels never fell to those observed in non-capacitated sperm. The authors suggested that since the inhibitors used were not perfectly specific for the ERK participants, it was unlikely that tyrosine phosphorylation would be completely eliminated. Nonetheless, using another battery of inhibitors, they investigated the possibility that the ERK pathway is influenced by cross-talk from other signaling pathways, including small Ras-like GTPase, B-RAF, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II, protein kinase C, and phosphatidylinositol 3-kinase. None of these alternative pathways appreciatively altered surface phosphotyrosine expression. Similarly, inhibition of the ERK cascade reduced the ability of the sperm to bind homologous ZP.



**FIGURE 3** Simplified cascade of signaling pathways reported to be involved in sperm capacitation. Major questions remain regarding how protein kinase A promotes the tyrosine phosphorylation of sperm proteins during capacitation. Both receptor and non-receptor tyrosine kinases may be involved.

The literature over the past 15 years indicates that PKA drives capacitation via sperm protein tyrosine phosphorylation. The link between the PKA pathway and some presumable PTK cascade is becoming clearer, with SRC tyrosine kinase being a very promising candidate. Although less studied than the PKA-mediated pathway, ERK signaling may be a parallel regulator of capacitation. Identification of the tyrosine phosphorylated kinase substrates provides molecular insight regarding the mechanisms and functional nature of capacitation. A summary of the major signaling molecules, putatively involved in capacitation, is presented in Figure 3.

## CONCLUSION

Capacitation is regulated by both intrinsic and extrinsic factors. Membrane cholesterol itself can be described as the ultimate ‘decapacitation factor’; its removal via components in the male and female tracts, and mediation of lipid raft behavior initiates the signal transduction pathways leading to capacitation. Calcium is essential to capacitation; it is presumably sequestered to the acrosome early in capacitation, and its mobilization occurs either later during capacitation or following ZP stimulation. Bicarbonate is particularly important in membrane hyperpolarization and for enabling calcium influx, increasing pHi and activating sAC, which stimulates a

PKA-driven PTK. Current studies suggest SRC to be a promising PTK candidate, although other pathways and/or kinases are likely involved. ERK activity seems to be a parallel albeit lesser characterized pathway associated with capacitation. Thanks to proteomic approaches, numerous capacitation-associated sperm tyrosine phosphoproteins can be identified, providing molecular insight regarding the mechanisms and functional nature of capacitation.

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