

The Molecular Basis of Sperm Capacitation

Review

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Testicular sperm that have undergone spermatogenesis and spermiogenesis appear mature from a morphological standpoint but have acquired neither progressive motility nor the ability to fertilize a metaphase II-arrested egg. In many species, progressive motility and fertilization competence are acquired during epididymal transit, but complete fertilization capacity *in vivo* is only gained upon residence in the female reproductive tract for a finite period of time. The molecular and physiological events that confer on the sperm the ability to fertilize during residence in the female tract are collectively known as "capacitation." These maturational events can also be accomplished *in vitro* in defined media, the composition of which approximates the environment of the female reproductive tract. Although capacitation was discovered independently by Austin (1951, 1952) and Chang (1951, 1955) nearly one-half century ago, little is known to date about the molecular basis of this important event. This brief review will consider some of the recent findings made toward an understanding of capacitation using *in vitro* models. The purpose of this review is not to provide an exhaustive analysis of capacitation but is to offer an

update and to discuss future avenues of research directed toward an understanding of the molecular basis of this event. We regret that we are unable to cite all of the important work that has led to the development of this field. Recent reviews by Florman and Babcock (1991), Storey and Kopf (1991), Cohen-Dayag and Eisenbach (1994), Yanagimachi (1994), and Harrison (1996) provide excellent supplementary reading.

Definition and Functional Assays of Capacitation

The definition of capacitation has been modified over the years to reflect many investigators' biases as to the physiological importance of this event. Although fertilization still represents the benchmark endpoint of a capacitated sperm, the ability of the sperm to undergo a regulated acrosome reaction (e.g., in response to the zona pellucida) can be taken as an earlier, upstream endpoint of this extratesticular maturational event. It must be stressed at this point that capacitation is also correlated with changes in sperm motility patterns in a number of species, designated as sperm hyperactivation (Yanagimachi, 1994; Suarez, 1996). There are experiments demonstrating the dissociation of capacitation and hyperactivation (Neill and Olds-Clarke, 1987), but it has not been conclusively demonstrated that hyperactivation of motility represents an event completely independent of capacitation (Suarez, 1996). Attempts to understand the process of capacitation at the molecular level, therefore, should include a consideration of events occurring both in the head (i.e., acrosome reaction) and in the tail (i.e., motility changes).

The oviduct or uterus represent the physiological sites of capacitation *in vivo* in many species (Yanagimachi, 1994). However, capacitation can be accomplished *in vitro* in numerous species by incubating cauda and/or ejaculated sperm under a variety of conditions in defined media that mimic the electrolyte composition of the oviductal fluid. In most cases, these media contain energy substrates, such as pyruvate, lactate, and glucose (depending on the species), a protein source (usually serum albumin), NaHCO_3 , and Ca^{2+} . The putative mechanism of action of these media components to promote capacitation at the molecular level is poorly understood and will be discussed in this review.

Since the ability of sperm to fertilize an egg is generally taken as the true endpoint of capacitation, and fertilization is a multistep process, simple and straightforward assays for capacitation have been difficult to develop. In fact, there are no direct assays of capacitation, and all of the assays to evaluate this process are based on different

Supported by NIH grants HD06274, HD34811, HD22732 and HD33052. X.P.N. and P.E.V. were supported by HD06274; H.G.H. was supported by USDA 9502560; M.F. and P.E.V. were supported by the Rockefeller Foundation; G.D.M. was supported by HD22732; J.L.B. was supported by HD06274.

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Received for publication December 1, 1997; accepted for publication December 8, 1997.

definitions. This, no doubt, is due in part to our ignorance regarding the molecular and physiological bases of this event. A thorough understanding of this event will be essential to permit the development of new and specific assays. The discussion below is not intended to cover the full spectrum of assays currently used to assess capacitation but to provide an overview of the advantages and disadvantages of the more common assays presently in use.

In Vitro Fertilization—One way to assess capacitation is to utilize *in vitro* fertilization assays. This technique has both advantages and disadvantages. An advantage is that the endpoint measured follows the classical definition of capacitation (i.e., sperm that are able to fertilize eggs have undergone capacitation). Although this technique is a very powerful tool to demonstrate that a particular incubation medium/condition is capable of capacitating sperm, it has obvious limitations for analysis if one wishes to examine whether a particular compound/incubation condition affects capacitation. For example, it cannot be assumed that if a specific compound/incubation condition inhibits *in vitro* fertilization that its mode of action is to inhibit capacitation, since fertilization is a multistep process that involves various aspects of sperm physiology (e.g., motility, acrosome reaction) as well as the interaction between gametes (e.g., zona pellucida binding, plasma membrane binding, and/or fusion). Moreover, the concentration of sperm used in the *in vitro* fertilization assays may dramatically impact interpretation of results since these assays normally use a much higher sperm:egg ratio than is normally encountered *in vivo*. Finally, it is also time-consuming and expensive to perform.

Induction of the Acrosome Reaction—An acrosome reaction induced by a physiologically relevant agent is considered to be an endpoint for the completion of capacitation (Florman and Babcock, 1991; Yanagimachi, 1994). This operational definition is supported by the assumption that sperm that undergo the acrosome reaction have already become capacitated. Numerous studies have demonstrated that either cauda epididymal or ejaculated sperm do not immediately possess the ability to undergo an acrosome reaction in response to biological agents, such as the zona pellucida or progesterone (Ward and Storey, 1984; Yanagimachi, 1994; Shi and Roldan, 1995; Visconti et al, 1995a). It is well accepted that these compounds induce the acrosome reaction only in sperm that are already capacitated (Florman and Babcock, 1991). The advantage of this definition (and the subsequent use of assays to assess this event) is that the acrosome reaction is closer timewise to capacitation than is fertilization. Moreover, acrosome reaction assays are easier to perform. A problem with these assays is that compounds that are able to stimulate or inhibit the acrosome reaction cannot be assumed to do so by stimulating or inhibiting capacita-

tion. Specifically, acrosome reactions might be able to occur in uncapacitated sperm when the cells are challenged with compounds that bypass capacitation.

Chlortetracycline Fluorescence—The antibiotic chlortetracycline (CTC) yields different patterns of distribution on the sperm surface that can be visualized as distinct fluorescence patterns depending on the capacitation and the acrosomal status of the sperm. These different patterns of CTC binding were first described in the mouse by Saling and Storey (1979), and the correlation of these patterns with the capacitation status of the sperm was subsequently defined by Ward and Storey (1984). Several investigators have used these patterns to assess capacitation in mouse sperm as well as in sperm from other species (Lee et al, 1987). It should be noted, however, that the distribution patterns in the sperm of other species are clearly different from those in the mouse (Lee et al, 1987); therefore, great care must be taken in the calibration of this assay for use in other species. The advantage of this method is that it monitors the capacitation status of the sperm independently of the acrosome reaction. The disadvantage of this method is that the mechanism by which CTC yields the different patterns is not clearly understood, and, therefore, the physiological/molecular events comprising capacitation that give rise to these patterns are completely unknown. It is believed that changes in the distribution of Ca^{2+} -CTC complexes bound to phospholipids in the plasma membrane are responsible for the different patterns observed. As a consequence, any compound that changes the fluorescent absorption spectrum of the CTC or of Ca^{2+} -phospholipid complexes or that quenches the fluorescence intensity of these complexes could potentially be interpreted as changing the capacitated state of the sperm. If this were to occur nonspecifically, interpretation of results using such an assay would be problematic.

Molecular Basis of Capacitation

The molecular basis of sperm capacitation is still poorly understood, although this biological phenomenon was first described close to 50 years ago. Although we are still quite far away from a complete understanding of this process, recent work by several laboratories is starting to lead to a unified hypothesis of how this event is controlled, and this is delineated in the working model outlined in Figure 1. The reader is referred to this figure throughout this review.

As stated previously, capacitation can occur *in vitro* spontaneously in defined media without the addition of biological fluids. Although this suggests that capacitation is intrinsically modulated by the sperm, such that these cells are preprogrammed to undergo capacitation when they are incubated in the appropriate medium, it does not rule out the influence of heretofore uncharacterized pos-

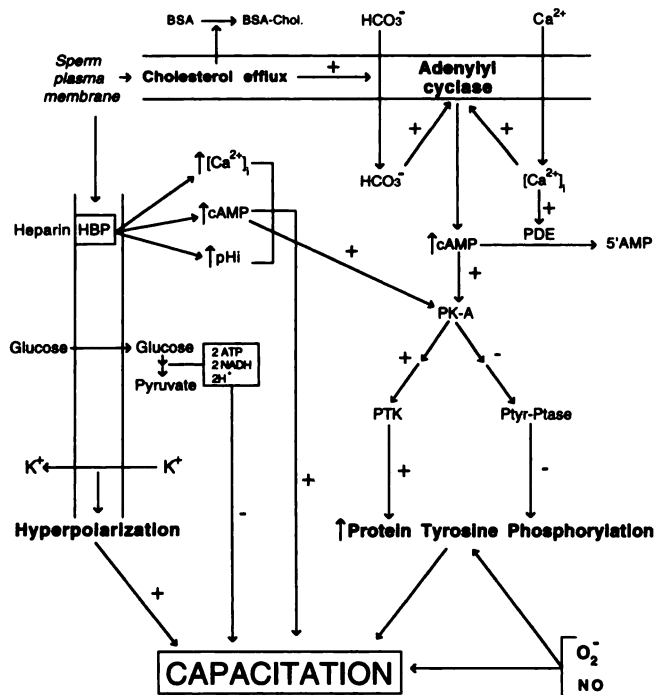


FIG. 1. Working model demonstrating the transmembrane and intracellular signaling pathways hypothesized to play a role in regulating sperm capacitation. This model is based on the work from a number of different laboratories cited in this review. (–) indicates negative regulation; (+) indicates positive regulation. Abbreviations used in this figure: BSA, bovine serum albumin; Chol, cholesterol; HBP, heparin-binding protein; PTK, protein tyrosine kinase; PTyr-Ptase, phosphotyrosine phosphatase; PDE, cyclic nucleotide phosphodiesterase; PK-A, protein kinase A.

itive/negative regulatory factors in the female reproductive tract. It is possible that the regulation of capacitation lies less in the stimulation of this process and more in the derepression of inhibitory modulators of capacitation through the removal of decapacitating factors (Hunter and Nornes, 1969; Yanagimachi, 1994). Although different media support capacitation in sperm from different species, it appears that certain components of the media, such as serum albumin, Ca^{2+} , and HCO_3^- , play an important regulatory role in promoting capacitation in all species studied thus far. Recent work is starting to clarify how these compounds are coupled to membrane, transmembrane, and intracellular signaling events regulating capacitation, and this will be considered below.

Role of Media Constituents in Capacitation In Vitro—Serum albumin present in the capacitation media (usually bovine serum albumin [BSA]) is believed to function during capacitation *in vitro* as a sink for the removal of cholesterol from the sperm plasma membrane (Go and Wolf, 1985; Langlais and Roberts, 1985; Cross, 1998). Removal of cholesterol could account for the membrane fluidity changes known to occur during capacitation (Wolf et al, 1986). It has not been established whether cholesterol re-

moval represents the only function of BSA, and little is known about its mechanism of action and the consequences of cholesterol removal on sperm membrane dynamics as it relates to capacitation. Experiments demonstrating that other cholesterol-binding proteins, such as high density lipoproteins (HDL), can replace albumin in *in vitro* fertilization assays (Thérien and Manjunath, 1996) suggest that the primary action of BSA may be in mediating cholesterol movement. Recently, Cross and co-workers (Cross, 1998) demonstrated that human semen contains cholesterol and that this sterol can account for the inhibitory effects of seminal plasma on human sperm capacitation, presumably by preventing cholesterol efflux from the sperm plasma membrane.

The involvement of Ca^{2+} in initiating and/or regulating capacitation is controversial at this time. In mouse sperm, there is evidence that extracellular Ca^{2+} is required for capacitation (Dasgupta et al, 1993; Visconti et al, 1995a), although these studies did not measure intracellular Ca^{2+} concentrations. An increase in intracellular sperm Ca^{2+} during capacitation has been described by some investigators, whereas others have shown that no changes occur during this maturational event (Yanagimachi, 1994). This ambiguity could be due, in part, to the well-demonstrated action of Ca^{2+} on the acrosome reaction and to the inherent difficulties in differentiating both of these events. However, as discussed below, the action of Ca^{2+} at the level of effector enzymes involved in sperm signal transduction (e.g., adenylyl cyclase, cyclic nucleotide phosphodiesterase) suggests that this divalent cation is likely to play an important role in capacitation.

The requirement of HCO_3^- for capacitation is well established in the mouse (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Shi and Roldan, 1995; Visconti et al, 1995a) and in the hamster (Boatman and Robbins, 1991), although it remains to be demonstrated in other mammalian species. Little is known about the mechanisms of HCO_3^- transport in sperm. The ability of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), well-known inhibitors of anion transporters, to block the actions of HCO_3^- on various sperm functions suggests that sperm contain anion transporters (Okamura et al, 1988; Visconti et al, 1990; Spira and Breitbart, 1992; Parkkila et al, 1993). It has been demonstrated that sperm contain a protein that is immunoreactive with an antibody to the AE1 class of anion transporters (Parkkila et al, 1993), but little is known about the identification and function of this protein in these cells. The transmembrane movement of HCO_3^- anions could be responsible for the known increase in intracellular pH that is observed during capacitation (Uguz et al, 1994; Zeng et al, 1996; Cross, 1998). An additional target for the action of this anion could be the regulation of sperm adenosine 3',5'-

cyclic monophosphate (cAMP) metabolism, since the mammalian sperm adenylyl cyclase is markedly stimulated by HCO_3^- by an unknown mechanism (Okamura et al, 1985; Garty and Salomon, 1987; Visconti et al, 1990, 1995b). From a physiological point of view, it is of interest that HCO_3^- concentrations are low in the epididymis and high in the seminal plasma and in the oviduct (reviewed in Harrison, 1996). Moreover, since HCO_3^- present in the extracellular milieu has also been positively correlated with the motility of pig sperm (Okamura et al, 1985), the HCO_3^- concentrations present in the male and female reproductive tracts could have an impact on capacitation. Specifically, the low levels of HCO_3^- in the epididymis would be conducive to maintaining sperm in an environment that does not support capacitation, whereas the higher concentrations of this anion in the female tract might contribute to capacitation.

Transmembrane and Intracellular Signal Transduction Regulating Capacitation In Vitro—The discussion of effectors and intracellular messengers mediating capacitation will be considered from two perspectives in this review. First, a discussion of the regulatory systems that appear to be common among different species, thereby forming a unifying hypothesis of capacitation, will be considered. Second, those regulatory processes that may be unique to one or more species then will be discussed and integrated into this unifying hypothesis where appropriate.

Although the role for cAMP in regulating mammalian sperm motility is well established, its role in capacitation, as well as in the acrosome reaction, is still uncertain (Yanagimachi, 1994). Our group, as well as others, has suggested a role for cAMP during capacitation (White and Aitken, 1989; Parrish et al, 1994; Visconti et al, 1995b; Leclerc et al, 1996), and we have recently demonstrated that protein kinase A (PK-A) activity increases during mouse sperm capacitation (Visconti et al, 1997). Measurement of PK-A activity represents the most accurate reflection of steady state changes in intracellular cAMP concentrations.

The mechanism by which cAMP concentrations are regulated during capacitation is also of great interest since the regulation of cAMP may be integrated with the aforementioned changes in Ca^{2+} and HCO_3^- movement. Both Ca^{2+} and HCO_3^- have been implicated in the regulation of sperm cAMP concentrations through their effects to stimulate adenylyl cyclase activity (Hyne and Garbers, 1979; Garty and Salomon, 1987). The mammalian sperm adenylyl cyclase possesses unique properties, and its regulation has been the subject of multiple studies. However, the sequence and topology of this enzyme has not yet been established, and the exact mechanism by which this enzyme is stimulated by these ions is not clear.

In attempts to further understand the signal transduc-

tion cascades that regulate capacitation, our laboratory has recently correlated mouse, human, and bovine sperm capacitation with an increase in protein tyrosine phosphorylation of a variety of substrates (Visconti et al, 1995a; Carrera et al, 1996; Galantino-Homer et al, 1997). Other labs have corroborated these results in these and other species (Aitken et al, 1995; Leclerc et al, 1996; Luconi et al, 1996; Emiliozzi and Fenichel, 1997). Using the mouse as an experimental paradigm, our laboratory demonstrated that capacitation *in vitro* of cauda epididymal sperm promotes the tyrosine phosphorylation of a subset of proteins of M_r 40,000–120,000. These phosphorylations are dependent on the presence of BSA, Ca^{2+} , and HCO_3^- in the medium, and the concentrations of these media constituents needed for protein tyrosine phosphorylation to occur are correlated with those needed for capacitation (Visconti et al, 1995a). Moreover, caput sperm, which do not possess the ability to undergo capacitation and to fertilize eggs (Yanagimachi, 1994), do not display these changes in protein tyrosine phosphorylation when incubated under conditions normally conducive to capacitation (Visconti et al, 1995a). The ability to display the changes in protein tyrosine phosphorylation are first seen during the caput-to-corpus transition (Fornes, Visconti, and Kopf, unpublished). These data suggest that the ability of mouse sperm to become capacitated, as well as their ability to undergo an increase in protein tyrosine phosphorylation, is acquired during epididymal transit and may represent an essential component of epididymal maturation in this species.

The absolute requirement for BSA, Ca^{2+} , and HCO_3^- in the extracellular medium to support protein tyrosine phosphorylation represents an interesting mode of regulation of the signal transduction cascade in sperm leading to these posttranslational modifications. As described previously, regulation of capacitation *in vitro* by BSA is thought to rely on its ability to serve as a sink for the removal of cholesterol from the sperm plasma membrane. This interrelationship between BSA and cholesterol movement also appears to be important in the regulation of protein tyrosine phosphorylation, since preloading BSA with a cholesterol analog to inhibit the ability of BSA to sequester sperm plasma membrane cholesterol inhibits protein tyrosine phosphorylation and sperm capacitation (Visconti, Ning, Fornes, Alvarez, and Kopf, unpublished). These, as well as other, experiments suggest that cholesterol release/movement is intimately tied to transmembrane signaling events in the sperm that ultimately regulate protein tyrosine phosphorylation. This novel mode of signal transduction clearly warrants further investigation.

The requirement of extracellular Ca^{2+} and HCO_3^- for both protein tyrosine phosphorylation and capacitation also represents a novel regulatory mechanism of cellular

signaling since these ions have been shown to be activators of the mammalian sperm adenylyl cyclase (Hyne and Garbers, 1979; Okamura et al, 1985; Garty and Salomon, 1987; Visconti et al, 1995b). Since there appears to be a relationship between Ca^{2+} , HCO_3^- , and increased adenylyl cyclase activity, experiments were designed to determine whether the action of these ions on protein tyrosine phosphorylation and capacitation involved a cAMP-mediated pathway. As previously stated, protein tyrosine phosphorylation does not occur when mouse sperm are incubated in the absence of BSA, Ca^{2+} , or HCO_3^- . However, incubating sperm in the absence of any of these compounds, but in the presence of cAMP agonists, results in an increase in protein tyrosine phosphorylation as well as capacitation (Visconti et al, 1995b). Moreover, protein tyrosine phosphorylation is accelerated by active cAMP agonists in complete media that support capacitation. Two major conclusions can be made from these experiments. First, the action of cAMP appears to be downstream of the actions of BSA, Ca^{2+} , and HCO_3^- but upstream of protein tyrosine phosphorylation. Second, protein tyrosine phosphorylation and capacitation are regulated through a PK-A pathway. Consistent with this hypothesis is the observation that two inhibitors of PK-A, Rp-cAMPS and H-89, both of which inhibit this enzyme by completely distinct mechanisms, inhibit both protein tyrosine phosphorylation and capacitation of sperm in complete medium (Visconti et al, 1995b). Moreover, PK-A activity increases during capacitation (Visconti et al, 1997). Since the mode of action of BSA appears to be tied to the removal of plasma membrane cholesterol, it is likely that cholesterol release is also upstream of the cAMP-induced protein tyrosine phosphorylation. Whether cholesterol removal is upstream or parallel to the action of Ca^{2+} and/or HCO_3^- is not presently known. One hypothesis to be tested is that the removal of cholesterol, with a resultant change in sperm plasma membrane fluidity, could modulate Ca^{2+} and/or HCO_3^- ion fluxes, leading to the activation of the adenylyl cyclase.

Taken together, these data suggest that protein tyrosine phosphorylation and capacitation appear to be under the regulation of a cAMP/PK-A pathway. Up-regulation of protein tyrosine phosphorylation by PK-A during sperm capacitation is, to our knowledge, the first demonstration of a connection between these signal transduction pathways at this level. Since similar results have now been reported in sperm of other species (Leclerc et al, 1996; Galantino-Homer et al, 1997), it is possible that this unique mode of signal transduction crosstalk may be universal to mammalian sperm. Presently, it is not known whether the increase in protein tyrosine phosphorylation is due to the stimulation of a tyrosine kinase, to an inhibition of a phosphotyrosine phosphatase, or to both. The nature of the regulatory enzymes in this unique signal

transduction pathway, as well as the identity of the phosphorylated substrates and their connection to capacitation, will remain an area of future research.

Intracellular pH (pH_i) regulates several aspects of mammalian sperm function, including capacitation. Although the transport mechanisms that control pH_i in these cells are not fully understood, two acid efflux mechanisms have been identified in mouse sperm (Zeng et al, 1996). One of these pathways shares the characteristics of the somatic cell Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger, and the second pathway does not require extracellular ions to function. These authors described an increase in pH_i during capacitation, and these data are consistent with reports by Vredenburg-Wilberg and Parrish (1995) describing an increase in pH_i during capacitation of bovine sperm by heparin. Although the increase in pH_i accompanying heparin-induced bovine sperm capacitation is not inhibited by Rp-cAMP (Uguz et al, 1994), this PK-A antagonist can block capacitation, suggesting that a PK-A regulatory pathway(s) functions either in parallel to, or downstream of, pathways activated as a consequence of changes in pH_i .

Hyperpolarization of the sperm plasma membrane has also been shown to accompany capacitation in mouse and bovine sperm (Zeng et al, 1995). Membrane hyperpolarization is due in part to an enhanced K^+ permeability and could be related to the release of inhibitory modulation during capacitation (Arnoult et al, 1996). Little is known about the consequences of this hyperpolarization; however, it is speculated that such membrane potential changes could recruit Ca^{2+} channels from an inactivated state to a closed, but activatable, state from which they could be subsequently opened by an agonist-induced depolarization (e.g., with the zona pellucida; Arnoult et al, 1996; Florman et al, 1998). Presently, the role of membrane potential in regulating any of the aforementioned aspects of capacitation at the molecular level is not known but remains an important avenue for future investigation.

The role of free radicals in sperm function has been studied by a number of different laboratories, and a majority of this work has focused on lipid peroxidation and sperm viability (Storey, 1997). However, more recent work using human sperm has focused on the role of superoxide anion generation related to capacitation and hyperactivation of motility (De Lamirande and Gagnon, 1993). Recently, Leclerc et al (1997) found that reactive oxygen species up-regulate protein tyrosine phosphorylation of several proteins. These results are in agreement with the work of Aitken et al (1995) who described an increase in protein tyrosine phosphorylation after stimulation of a postulated endogenous NADPH-oxidase or after addition of H_2O_2 . Presently, it is not known how free radical generation leads to capacitation. Moreover, the localization of the free radical generating system(s) in

sperm, as well as whether the action of superoxide anion is dependent or independent of cAMP, is not presently known.

Studies of bovine sperm capacitation have shown that capacitation *in vitro* can be accomplished in media containing either heparin (Parrish et al, 1988) or oviductal fluid (in which the active capacitating agent is thought to be a heparin-like glycosaminoglycan). Heparin (or glycosaminoglycans) does not appear to be essential for capacitation in any of the other species studied thus far. However, it should be emphasized that, since most studies are performed *in vitro*, one cannot rule out the possibility that glycosaminoglycans associated with the female tract or the cumulus-enclosed oocyte play an important role in capacitation *in vivo*. It is thought that glycosaminoglycans may promote capacitation by binding to and removing seminal plasma proteins that are adsorbed to the sperm plasma membrane and are normally thought to function to inhibit capacitation (Miller et al, 1990; Thérien et al, 1995). Interestingly, heparin also increases cAMP synthesis (Parrish et al, 1994), elevates pH_i (see above), and regulates the capacitation-associated changes in protein tyrosine phosphorylation (Galantino-Homer et al, 1997). The mechanism by which this occurs, and its physiological relevance, is not clear.

The issue of whether glucose has inhibitory or stimulatory actions on capacitation is controversial and is apparently species dependent. Glucose inhibits heparin-induced bovine sperm capacitation *in vitro* by a mechanism involving effects on cAMP metabolism and a reduction of pH_i (Parrish et al, 1994; Uguz et al, 1994). The capacitation-associated increase in protein tyrosine phosphorylation in bovine sperm incubated in media containing heparin is also inhibited by glucose (Galantino-Homer et al, 1997). We have observed that, although glucose has these inhibitory effects on protein tyrosine phosphorylation in bovine sperm, capacitation media for mouse sperm, which contains glucose, has no apparent inhibitory effects on protein tyrosine phosphorylation (Visconti et al, 1995a). Paradoxically, others have found that glucose is beneficial for capacitation in other species (Fraser and Herod, 1990; Rogers and Perreault, 1990; Mahadevan et al, 1997). The species-dependent differences in responses to this saccharide are not understood, nor is its mechanism of action.

Summary

Work emanating from several laboratories is adding to our knowledge of the molecular basis of sperm capacitation, leading to a unified model of this event. Over the next few years, several questions of considerable importance must be addressed. First, what is the mechanism by which cholesterol moves from the sperm plasma membrane, and how does this movement initiate intracellular signaling?

Second, what is the mechanism by which the cAMP/PK-A pathway is stimulated, and how does stimulation of this pathway lead to crosstalk and up-regulation of protein tyrosine phosphorylation? Finally, what is the identity of the substrates that are phosphorylated on tyrosine residues, and how does the phosphorylation of these substrates impact on the major endpoints of capacitation (e.g., hyperactivation of motility, competence to undergo a regulated acrosome reaction, and fertilization)? Answers to such questions may provide us with a molecular insight into this poorly understood, but extremely important, extratesticular maturational event.

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