[Frontiers in Bioscience 11, 1636-1645, May 1, 2006]

Regulation of mammalian sperm capacitation by endogenous molecules

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1. ABSTRACT

Capacitation *in vitro* in mammalian spermatozoa can be regulated by a number of first messengers, including fertilization promoting peptide, adenosine, calcitonin and angiotensin II, all of which are found in seminal plasma. The responses appear to involve several separate signal transduction pathways that have a common end point. These seminal-plasma derived first messengers can bind to specific receptors and directly or indirectly modulate the activity of membrane-associated adenylyl cyclase isoforms and production of the second messenger cAMP. Responses to all of these except angiotensin II involve initial acceleration of cAMP production and capacitation followed by inhibition of both cAMP production and spontaneous acrosome loss, resulting in maintenance of fertilizing potential. Appropriate G proteins and various phosphodiesterase isoforms also appear to be involved. The transition from stimulatory to inhibitory responses involves loss of decapacitation factors (DF) from receptors (DF-R) on the external surface; a DF-R present on both mouse and human spermatozoa has recently been identified as phosphatidylethanolamine-binding protein 1. The presence/absence of DF appears to cause changes in the plasma membrane that then alter the functionality of various membrane-associated proteins, including receptors. Since spermatozoa contact these first messengers at ejaculation, it is plausible that their actions observed *in vitro* also occur *in vivo,* allowing these molecules to play a pivotal role in enhancing the chances of successful fertilization.

2. INTRODUCTION

In 1998 a review on the role of fertilization promoting peptide (FPP) in modulating mammalian sperm function was published in *Frontiers in Bioscience* (1). There was evidence that FPP, a peptide found in nanomolar quantities in seminal plasma, could modulate the adenylyl cyclase (AC)/cAMP signal transduction pathway. Experimental results indicated that the FPP receptor TCP11 appeared to interact with adenosine receptors in order to elicit responses; adenosine receptors are G protein-coupled (GPCRs) and are known to alter the function of ACs in somatic cells (2). In the relatively short time since the publication of that review, investigation of other small molecules also found in seminal plasma has provided evidence that mammalian sperm have several different G protein-modulated signal transduction pathways that affect AC and its production of the second messenger cAMP, indicating that these molecules all fit the definition of 'first messengers'. During that same time period, other studies systematically investigated the various components of relevant signalling pathways and obtained new and novel information about G proteins, membrane-associated AC (mAC) isoforms and cyclic nucleotide phosphodiesterase (PDE) isoforms. Finally, even more recent results obtained in a study of decapacitation factors, the loss of which is known to be a key component of capacitation, have shed new light on mechanisms involved in capacitation that lead to altered functionality of GPCRs.

Why are these findings important? When spermatozoa are released from the male tract, they are not yet able to fertilize: they require some species-dependent time during which they 'switch on' physiologically and become able to interact with an oocyte. Austin (3) called this change in fertilization competence 'capacitation', signifying acquisition of the capacity to fertilize. When spermatozoa are studied *in vitro*, their release into an appropriate culture medium promotes initiation of capacitation. Generally the changes continue until cells have capacitated, but some cells will not stop and so will 'over capacitate' and undergo spontaneous acrosome loss. This results in loss of the plasma membrane over the distal portion of the sperm head and consequent loss of special molecules needed for binding to the zona pellucida, a necessary prerequisite to successful fertilization. The fact that endogenous molecules, normally encountered by mammalian spermatozoa prior to meeting an oocyte, can bind to the cells and regulate capacitation, allowing them to capacitate but not to undergo spontaneous acrosome loss, could play important roles in maintaining sperm fertilizing potential *in vivo*.

3. OTHER FIRST MESSENGERS IN SEMINAL PLASMA AND THEIR SPECIFIC RECEPTORS ON SPERMATOZOA

The first studies on FPP showed a biphasic pattern of response, initially stimulatory and then inhibitory. Because adenosine is known to be present in seminal plasma (4) and previously had been shown to have a capacitation state-dependent biphasic effect on production of cAMP in mouse spermatozoa (5), adenosine was assessed using the FPP protocols and shown to elicit the same responses (6). Stein et al (5) had hypothesized that spermatozoa have both stimulatory and inhibitory adenosine receptors that were responsible for the regulation of cAMP production they had detected and so evidence to support this hypothesis was sought. Several different subtypes of adenosine receptors have been identified in somatic cells, with some stimulating and others inhibiting AC/cAMP (2). By using specific agonists and antagonists, it was possible to demonstrate that both stimulatory A_{2A} and inhibitory A_1 adenosine receptors are present on mouse spermatozoa, but the former function only in uncapacitated spermatozoa while the latter function only in capacitated cells (7). This means that reagents able to act only at A_{2A} receptors have significant effects on uncapacitated spermatozoa but no effect on capacitated cells, while agonists/antagonists acting only on A_1 receptors have effects on capacitated cells but none on uncapacitated cells.

Since adenosine receptors are GPCRs, involvement of different G proteins in the observed responses would be predicted, stimulatory G proteins interacting with A_{2A} receptors and inhibitory G proteins with A_1 receptors. Subsequent experimental results obtained using bacterial toxins support this hypothesis. Cholera toxin, known to irreversibly activate stimulatory G alpha subunits, accelerated capacitation in uncapacitated mouse sperm suspensions but had no effect on capacitated cells, consistent with involvement of G alpha s in the stimulatory responses to adenosine and to FPP. In contrast, pertussis toxin, known to irreversibly inactivate several G alpha i/o subunits, blocked responses to both adenosine and FPP in capacitated suspensions but had no effect on uncapacitated suspensions (7). Further investigations demonstrated that cholera toxin, FPP and an A_{2A} receptor agonist all stimulated protein tyrosine phosphorylation in uncapacitated mouse sperm suspensions, responses often cited as signifying a stimulation of capacitation (8). In contrast, FPP inhibited phosphorylation in capacitated cells but inclusion of pertussis toxin blocked this inhibition, again consistent with the involvement of appropriate G proteins (9). Inhibition of phosphorylation thus appears to be correlated with the inhibition of spontaneous acrosome loss and consequent maintenance of fertilizing potential.

Immunolocalization studies confirmed the presence of two populations of adenosine receptors, both being found on the acrosomal cap and along the flagellum, especially the principal piece. Interestingly, the fluorescence signal observed on the head was much stronger on cells in the capacitation state where the specific receptors are known to be active. Thus staining for the stimulatory receptors was much stronger on heads of uncapacitated cells while that for the inhibitory receptors was much stronger on capacitated spermatozoa (Figure 1; 10). This presumably reflects changes in receptor conformation such that the antigenic epitopes are more or less accessible to the specific antibodies. In addition, there are also alterations in the accessibility of adenosine binding sites on the receptors that affect their functionality. The presence of adenosine receptors on the same regions of the cell where TCP11, receptor for FPP, is found (11) supports our hypothesis that FPP receptors somehow interact with adenosine receptors to allow responses to FPP.

Following initial investigations of FPP and adenosine, subsequent studies focused on other small molecules with which spermatozoa would routinely come into contact at ejaculation. Like FPP, calcitonin is also a peptide found in seminal plasma; although usually considered to function in bone homeostasis, there is considerably more calcitonin in human seminal plasma than in blood plasma (12). Experiments to determine whether calcitonin has any detectable effect on sperm function revealed that low nanomolar concentrations elicit the same responses as FPP and adenosine, i.e., significantly accelerating capacitation and then inhibiting spontaneous acrosome loss in both mouse and human spermatozoa as shown by chlortetracycline (CTC) analysis and *in vitro* fertilization IVF (13,14). Like FPP, calcitonin acts on AC/cAMP, first significantly stimulating cAMP production and then significantly inhibiting it but the change from stimulation to inhibition of cAMP production in response to calcitonin takes longer than that elicited by FPP (15). Thus these two peptides reached the same endpoints, but at different rates.

Immunolocalization of calcitonin receptors, also GPCRs, revealed them to be present on both the acrosomal cap region and the flagellum of mouse spermatozoa, with no evidence for two different populations of calcitonin

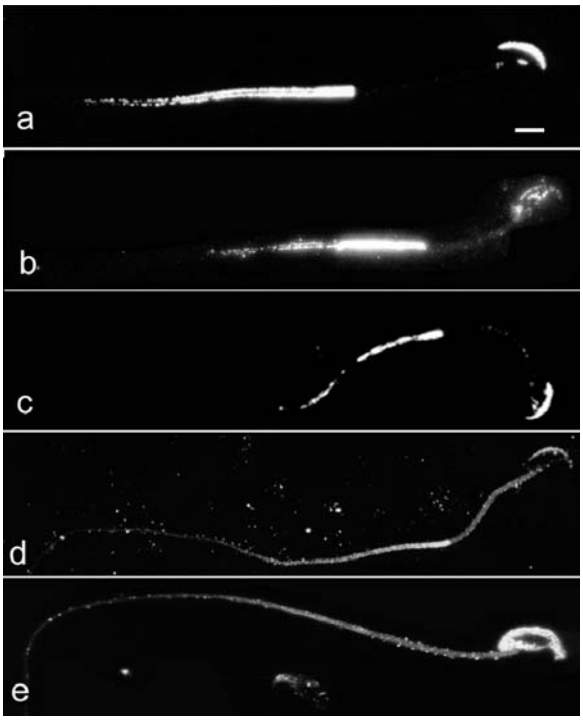

Figure l. Immunolocalization of DF-R/PEBP 1 on mouse spermatozoa. Uncapacitated, DF-depleted (experimentally capacitated by centrifugation to remove DF) and naturally capacitated suspensions (incubation for ~2 h) were evaluated +/- addition of crude DF or treatment with PI-PLC to remove GPI-anchored proteins. The amount of fluorescence was capacitation-state dependent; uncapacitated cells had very bright fluorescence in the acrosomal cap region but less fluorescence in the postacrosomal region and the flagellum. Capacitated spermatozoa had brighter fluorescence in both the postacrosomal region and the flagellum than seen in uncapacitated cells, while the acrosomal cap fluorescence was less than that seen prior to capacitation. After addition of crude DF to capacitated suspensions, the fluorescence pattern reverted to that seen in uncapacitated cells. Treatment with PI-PLC prior to staining resulted in almost no fluorescence. The bar represents 10 μm (Reproduced from 46 with permission).

receptors. Having a single receptor type may contribute to the longer time needed for calcitonin to trigger the change from stimulatory to inhibitory effects on AC/cAMP than for FPP; responses to the latter appear to involve two different adenosine receptor populations which may allow more rapid switching to occur. Consistent with the receptors being GPCRs, the inclusion of pertussis toxin blocked effects of calcitonin in capacitated spermatozoa (13). It was also found that calcitonin, unlike FPP, can elicit positive responses in Ca^{2+}-deficient medium, significantly accelerating capacitation and stimulating cAMP production, while FPP cannot (15). This suggests that these two first messengers may be acting on different ACs.

Since mammalian spermatozoa would encounter these first messengers from the time of ejaculation onwards, their ability to regulate AC/cAMP in a biphasic manner could be of considerable importance *in vivo*. Biologically, both the stimulatory and inhibitory phases are noteworthy but we think that the second phase, involving inhibition of cAMP production and consequent inhibition of spontaneous acrosome loss, is particularly important because this helps to maintain spermatozoa in a potentially fertilizing state for some considerable time. For example, we were able to show that inhibition of human sperm acrosome loss by a combination of first messengers was maintained for at least 3 h (14). We also have demonstrated (7) that the concentration of FPP needed to block the acrosome reaction (6.25 nM) is much lower than that required to promote acceleration of capacitation (~50-100 nM for maximal response).

Angiotensin II (AII) is yet another peptide present in seminal plasma at a higher concentration than that found in blood (16) and it, too, has important physiological effects on capacitation. However, unlike FPP, adenosine and calcitonin, AII has been shown to accelerate capacitation but not to inhibit spontaneous acrosome loss in both mouse and human spermatozoa (13,14); this is associated with continuous stimulation of cAMP production and protein tyrosine phosphorylation (17). In uncapacitated spermatozoa, AII stimulated phosphorylation within 20 min in both the acrosomal cap and principal piece, while in capacitated cells it stimulated phosphorylation primarily in the whole of the flagellum, with some signal in the equatorial segment. Interestingly, when AII was tested in combination with FPP and/or calcitonin on capacitated suspensions, the ability of the latter to inhibit acrosome loss was able to over-ride the actions of AII in both mouse and human spermatozoa (13,14). AII receptors are also GPCRs and when pertussis toxin was included, it blocked responses to AII in both uncapacitated and capacitated suspensions as determined by CTC analysis and cAMP production. This was puzzling because pertussis toxin had no effect on responses to FPP in uncapacitated spermatozoa but did block responses in capacitated cells. Further investigation confirmed that pertussis toxin does indeed have different effects on responses to FPP and to AII, suggesting that the initial step in response to these two first messengers is different for the two peptides. Since both stimulate cAMP production in uncapacitated suspensions, it seems likely that binding of AII to its receptor stimulates cAMP in an indirect manner, possibly by altering intracellular Ca^{2+} concentration (18). Immunolocalization studies revealed that AII receptors ,like the receptors for FPP, adenosine and calcitonin, are located on both the acrosomal cap and along the flagellum of mouse and human spermatozoa (17).

4. G PROTEINS

Evidence obtained in several earlier studies had suggested that mammalian spermatozoa *do* have inhibitory G alpha subunits but *do not* have stimulatory G alpha subunits (19-22). However, there were reasons to think that spermatozoa might well have both types of G alpha subunits rather than just inhibitory ones. Firstly, as mentioned earlier, physiological and biochemical evidence indicated the presence of two populations of adenosine

receptors (5, 7) and stimulatory A_{2A} are known to work via G alpha s in somatic cells (2). Secondly, the positive responses to cholera toxin suggested the presence of G alpha s (7, 9). Using mouse sperm membrane preparations and commercially available antibodies, Western blotting identified G alpha s (primarily the long isoform of ~48 kDa) and several different inhibitory G alpha subunits, including G alpha i2, i3 and o, but not G alpha i1 (7). More recently, Western blotting of mouse and human sperm lysates also identified G alpha s, the most abundant isoform again being G alpha s-long, ~48 kDa, rather than ~45 kDa G alpha s-short, the isoform most often found in somatic tissues. In addition, cholera toxin enhanced the ADP ribosylation of an appropriately-sized protein (~48 kDa) and significantly stimulated cAMP production in mouse sperm preparations (23). In support of those findings, Spehr and colleagues (24) have recently reported detecting the long isoform of G alpha s in human spermatozoa using multidimensional protein identification technology.

Immunolocalization studies revealed the presence and specific locations for G alpha s, olf, i2, i3, o and q/11 in both mouse and human spermatozoa (23). Both the stimulatory G alphas s and olf, as well as the inhibitory G alpha i2, were found in the acrosomal cap region and the flagellum, the same regions as the various receptors for the first messengers discussed above. This location would be consistent with their involvement in signal transduction pathways involving the various GPCRs discussed above. Recent investigations into possible odorant receptor-mediated chemotactic signalling pathways in spermatozoa have also identified the presence of G alpha olf in human spermatozoa (24).

5. ADENYLYL CYCLASE ISOFORMS

To date nine isoforms of membrane-associated AC (mAC), all G protein-regulated and able to respond to a variety of activating molecules, have been identified in somatic cells (25). There is also a soluble isoform (sAC), structurally unrelated to mACs, that has been found particularly in testicular tissue and in spermatozoa (26). The predominant form of sAC is ~48 kDa, considerably smaller than mACs found in somatic cells (>100 kDa); it is stimulated by bicarbonate, Ca^{2+} and Mn^{2+} but is not regulated by G proteins and does not respond to known modulators of mAC (26,27).

Although there have been some claims that sAC is the only AC of importance in sperm physiology, a number of recent studies have provided strong evidence for the presence and function of mACs as well. For example, biochemical experiments showed that both fluoride and forskolin stimulate production of cAMP in mouse spermatozoa, with responses to forskolin being inhibited significantly in the presence of dideoxyadenosine (ddAdo), a specific inhibitor that acts on the P site of mAC. In addition, physiological experiments using CTC analysis showed that forskolin stimulated both capacitation and acrosome loss, consistent with this compound continuously stimulating mAC/cAMP; however, the inclusion of ddAdo inhibited these responses (28). There is also extensive evidence that cholera and pertussis toxins, known to act on stimulatory and inhibitory G proteins, respectively, and various GTP analogues can significantly alter cAMP production in mammalian spermatozoa (29). In addition, the various first messengers discussed above all bind to receptors located on the sperm surface and regulate AC/cAMP. These results suggest that the responses in question involve mACs and not sAC, which is reported to be non-responsive to known mAC and G protein modulators (26,27).

It seems likely that both mACs and sAC contribute to mammalian sperm cAMP production. Recent studies have provided evidence that bicarbonate, presumably acting on sAC, plays a role early in capacitation. Within 15 min of bicarbonate introduction, marked alterations in the sperm membrane lipid architecture, due to cAMP-dependent phosphorylation, can be detected (30). It is plausible that these membrane modifications might then alter the conformation and hence functionality of the mAC isoforms and/or GPCRs known to regulate mAC activity.

At present, very little is known about the relative contributions of sAC and mAC to the promotion of capacitation, *in vitro* or *in vivo*. However, the presence of bicarbonate throughout the female tract would probably keep sAC in an active state, continuously producing cAMP, and unregulated cAMP accumulation could result in spontaneous acrosome reactions and loss of fertility. In contrast, the mACs are subject to modulation by external regulatory molecules that bind to specific receptors on the cell surface; these molecules are encountered at ejaculation and are probably also present in the female tract. Studies of mammalian spermatozoa *in vitro,* in bicarbonate- and Ca^{2+}-containing medium that would promote continuous activity of sAC, show that total cAMP production can be significantly altered by the introduction of the first messengers discussed earlier. These results thus suggest that mACs can make an important contribution to total cAMP and that they can play an effective role in *regulating* cAMP levels, even when sAC is active. Biologically, this could be very important *in vivo* because inhibition of mAC inhibits spontaneous acrosome loss and so preserves sperm fertilizing ability. Therefore, it seems likely that both sAC and mACs contribute to cAMP production needed for capacitation. Consistent with that hypothesis, male mice with targeted disruption of the sAC gene produce spermatozoa with badly impaired motility and are infertile (31), while male mice with targeted disruption of the mAC3 gene produce spermatozoa with faulty motility and a significantly higher incidence of spontaneous acrosome reactions than $mAC3^{+/+}$ males and are very subfertile (32).

Until recently there has been relatively little information about which mAC isoforms might be present in mammalian spermatozoa, the exception being a report that mAC3, the isoform that functions in the olfactory signalling pathway, had been found in immature male germ cells (33,34). However, a new study has found evidence for the presence of several mAC isoforms in mature mouse spermatozoa, with mAC2, 3 and 8 being relatively

Table 1. Distribution of the most abundant isoforms of cyclic nucleotide phosphodiesterase (PDE) found in mouse spermatozoa using isoform-specific antibodies

PDE Isoform	Location
1A	Midpiece +, principal piece ++
4D	Acrosome ++, neck ++, principal piece ++, midpiece +
6α	Ventral head surface ++, whole flagellum ++, rest of head +
8A	Midpiece ++
10A	Acrosomal cap ++, equatorial segment +, flagellum ++
11A	Acrosomal cap ++, flagellum +

++: s strong/moderate signal; +: weak signal. Adapted with permission from reference 35

abundant and mAC1 and 4 being less abundant (28). Immunolocalization results have shown that these mAC isoforms have distinct locations in the acrosomal and flagellar regions. In particular, mAC3 and mAC8 were found in both the acrosomal cap and flagellar regions, the same locations where the receptors and G proteins discussed above are also found; a recent study reported a similar location for mAC3 in human spermatozoa (24).

Since the various mAC isoforms differ in their requirements for functioning (25), the presence of multiple mAC isoforms in mammalian spermatozoa would allow the various first messengers and their specific receptors to modulate cAMP via different mACs. This would seem to be the case for adenosine and calcitonin receptors, since the former require added Ca^{2+} to be present before biological responses can be detected yet the latter show significant function in the absence of added Ca^{2+} (15). Finally, Western blotting of mouse sperm lysates under conditions that would minimize proteolytic activity revealed that the mAC proteins detected with specific antibodies were considerably smaller (~50-60 kDa) than somatic cell mACs (>130 kDa), suggesting that mAC isoforms in spermatozoa may function as shorter forms than those found in other cells (28).

6. PHOSPHODIESTERASES

The amount of cAMP available within spermatozoa will depend on the relative activities of both ACs, enzymes producing cAMP, and cyclic nucleotide phosphodiesterases (PDEs), enzymes metabolizing cAMP to 5' AMP. Thus far, knowledge about PDEs in spermatozoa is rather limited and new isoforms of PDE are still being identified. A number of years ago, we observed that PDE activity decreased as spermatozoa underwent capacitation; this could promote a gradual rise in cAMP and so stimulate various events within the cells (35), but which PDEs might be involved? To date, 11 PDE families, each differing in structure, substrate preference, kinetics and sensitivity to inhibitors, have been identified. We have recently reported the presence of several different PDE isoforms in mature mouse spermatozoa and provided evidence that at least some may play roles during capacitation (36). The PDE proteins identified included PDEs 1, 4, 6, 8, 10 and 11. In immunolocalization studies, PDE 1A was present mainly in the flagellum and PDE 11A appeared to quite abundant in the acrosomal region, but PDEs 4D and 10A were present in both the acrosomal region and the flagellum, the same regions where various receptors, mACs and G proteins have been found (see Table 1). Thus those last two PDEs are appropriately sited to interact with the signal transduction pathways discussed above. Consistent with the localization data, both MMPX, a specific inhibitor of PDE 1, and rolipram, a specific inhibitor of PDE 4, significantly increased cAMP levels in live mouse spermatozoa, confirming that both isoforms are present and active. Another interesting result was that first messengers such as CGS 21680 (an A_{2A} adenosine receptor agonist), calcitonin and FPP not only stimulated cAMP production but also appeared to cause a decrease in PDE activity, a response which would contribute to a further increase in cAMP within the cells.

Of the various isoforms, PDEs 1 and 4 are the two that have been most often linked with mammalian sperm function; in some studies the suggested link has been based on effects of specific PDE inhibitors and in others it has been based on immunolocalization data. One study found that use of a specific PDE 1 inhibitor at a relatively high concentration had no effect on human sperm motility but did stimulate the acrosome reaction within 30 min (37). This is a puzzling result for two reasons. Firstly, studies on mammalian spermatozoa have found PDE 1 to be located primarily in the flagellum and not in the acrosomal region (36,38). Secondly, it is unlikely that a major effect on capacitation and acrosome loss would occur within 30 min of adding a PDE inhibitor to uncapacitated suspensions, especially since human spermatozoa generally require several hours to capacitate (14). The location of PDE 1 suggests it would be more likely to affect motility than acrosome loss, whereas PDE 4 potentially could affect both. Indeed, Baxendale and Fraser (36) showed that, although incubation of uncapacitated mouse spermatozoa in the presence of two different PDE inhibitors resulted in increased cAMP levels, the ability of the inhibitors to affect capacitation in live intact cells was very different. MMPX, which acts on PDE 1 found mainly in the flagellum, failed to significantly stimulate either capacitation as determined by CTC analysis or fertilizing ability as determined by IVF. In contrast, rolipram, which acts on PDE 4 that is located in both the head and the flagellum, significantly accelerated capacitation and significantly stimulated *in vitro* fertilizing ability.

It is worth pointing out that although rolipram accelerated capacitation within a short time (~30 min), it did not trigger the acrosome reaction as well. Current evidence suggests that cAMP must reach a threshold level in order to trigger the acrosome reaction. PDE inhibitors cause a rise in cAMP by inhibiting the breakdown of existing cAMP and, under the conditions used in these

experiments, cAMP production would be less than that observed in spermatozoa treated with first messengers such as calcitonin and FPP. Therefore, it would take longer than 30 min to reach the cAMP threshold needed to induce the acrosome reaction. Overall, these results suggest that PDE location within the cell determines whether the enzyme can significantly affect sperm function. They also suggest that at least some PDEs could play roles that would affect capacitation and general motility.

7. INVOLVEMENT OF DECAPACITATION FACTORS

It has been known for over 50 years that capacitation plays a crucial role in successful fertilization: failure to complete capacitation will result in failure to fertilize. Despite its importance, the molecular events of capacitation are still poorly understood but it is generally acknowledged that an important component involves changes in the sperm surface, including loss, unmasking or rearrangements of various proteins (39). The molecules that are lost during capacitation are frequently referred to as 'decapacitation factors' (DF; 40) because the addition of DF to capacitated, fertile suspensions will cause them to revert rapidly to the uncapacitated, non-fertilizing state. However, with continued incubation the DF will be lost and the spermatozoa will regain fertilizing ability, thus showing that capacitation, unlike the acrosome reaction, is reversible. In some species DF is present on spermatozoa in the epididymis but in other species the DF is present in seminal plasma and so only associates with spermatozoa after ejaculation (39).

For over two decades we have been studying a DF that is present on epididymal mouse spermatozoa. It can be removed from uncapacitated cells by gentle centrifugation, resulting in highly fertile suspensions, while its addition to capacitated suspensions results in reversion to the uncapacitated, non-fertilizing state; with time, the cells will re-capacitate (41). Current evidence indicates that this DF is a protein of ~40 kDa, retains biological activity even after boiling and has carbohydrates that contribute to biological function (42). One consequence of DF binding to mouse spermatozoa appears to be stimulation of a calmodulin-sensitive Ca^{2+}-ATPase, leading to maintenance of low intracellular concentrations of Ca^{2+}; immunolocalization studies suggested that the calcium pump is located particularly in the postacrosomal region of the sperm head (43). The gradual loss or inactivation of DF during capacitation would result in decreasing activity of the Ca^{2+}-ATPase and a consequent rise in intracellular Ca^{2+} that then could stimulate events within the spermatozoon needed for acquisition of fertilizing ability.

Initially this DF was thought to bind directly to the sperm plasma membrane but this proved to be incorrect. Treating suspensions with phosphatidylinositol-specific phospholipase C (PI-PLC) to cleave glycosylphosphatidylinositol (GPI) anchors resulted in significant loss of DF and consequent advancement to the capacitated state, as determined using CTC fluorescence. However, addition of DF to PI-PLC-treated suspensions caused no reversion to the uncapacitated state, suggesting that DF could no longer bind to these cells (44). Thus another molecule, the DF receptor (DF-R), appears to be the one bound to the sperm surface. Further experiments revealed that DF has fucose moieties and DF-R has complementary fucose-binding sites; the importance of these interactions is demonstrated by the ability of exogenous fucose to quickly displace endogenous DF from uncapacitated spermatozoa and produce highly fertile suspensions (44). Furthermore, these molecules and their mechanism of action are not restricted to mouse spermatozoa. Evidence from many mammalian species has supported the involvement of a Ca^{2+}-ATPase in capacitation and the mouse sperm DF can act on human, as well as mouse, spermatozoa. Addition of mouse DF to capacitated human sperm suspensions for 1 hour caused a significant reversion to the uncapacitated state, determined by CTC (45) and addition of exogenous fucose to human sperm suspensions accelerated capacitation (14), suggesting the presence in human spermatozoa of proteins similar to mouse DF and DF-R.

Very recently we achieved fucose-based affinity purification of a ~23 kDa protein from PI-PLC-treated mature mouse spermatozoa that has DF-R biological activity. Sequencing results show high homology with phosphatidylethanolamine binding protein 1 (PEBP 1); the DF-R peptide fragments, representing ~76% of the whole PEBP 1 sequence, have 100% homology when aligned with the published sequence and so we have proposed that DF-R is PEBP 1 (46). Although PI-PLC treatment releases DF-R/PEBP 1 from the sperm surface, the protein has no clear GPI attachment site, suggesting that DF-R may be attached to a GPI-anchored protein rather than being attached directly to the sperm plasma membrane via a GPI moiety.

Following cloning and bacterial expression, recombinant PEBP 1 had the biological activity of DF-R, being able to remove DF activity in solution when used at concentrations as low as 1 nM; in contrast, recombinant PEBP 2, a closely related protein, had no detectable activity, even when used at 600 nM. A polyclonal antiserum directed against PEBP 1 recognized recombinant PEBP 1 and a protein in mouse and human sperm lysates of the correct size, but did not recognize PEBP 2. Immunocytochemical localization experiments using both mouse and human sperm have provided further important information, revealing that DF-R/PEBP 1 is located on both the head (acrosomal cap and postacrosomal regions) and the flagellum. Furthermore, it can be removed by treating live cells with PI-PLC prior to immunocytochemistry, consistent with a surface location. Most intriguingly, however, the intensity of fluorescent signal observed is both capacitation state-dependent and reversible, consistent with the known reversibility of capacitation; these results suggest that the DF-R conformation can change and so alter the accessibility of the antigenic epitope (Figure 2).

Although our earlier work showed that the presence/absence of DF resulted in altered Ca^{2+}-ATPase activity (43), recent results (46) indicate that DF binding to spermatozoa has more wide-ranging effects, consistent with

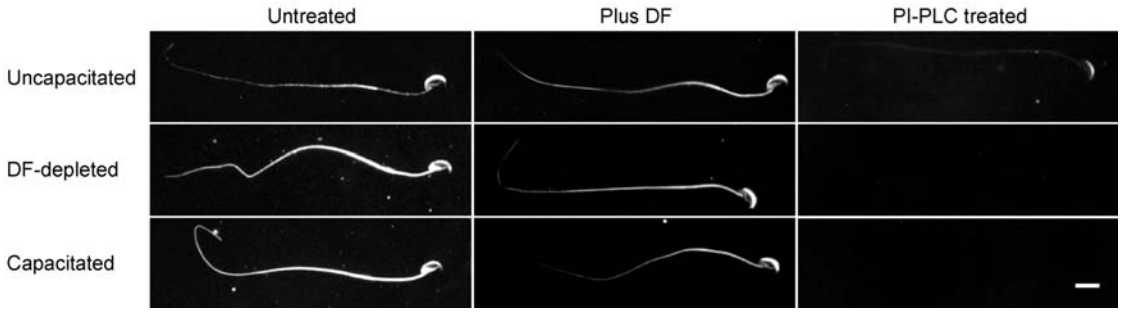

Figure 2. Immunolocalization of adenosine receptors on uncapacitated and capacitated mouse spermatozoa. Stimulatory A_{2A} adenosine receptors are present on both the acrosomal cap and the flagellum, but fluorescence is more intense in uncapacitated (a) than in capacitated (b) spermatozoa. A brief incubation of capacitated cells with purified decapacitation factor caused the fluorescence to revert to the pattern seen in uncapacitated spermatozoa (c). Inhibitory A_1 adenosine receptors have a similar distribution, but the fluorescence is noticeably more intense in capacitated (e) than in uncapacitated spermatozoa (d). The bar represents 5 μm (Reproduced from 10 with permission).

Table 2. Signal transduction pathway components and modulators that are located on both the acrosomal cap region and the flagellum

Receptors	**Action**	**References**
• A_{2A} adenosine	Stimulatory	10
• A_1 adenosine	Inhibitory	10
• Angiotensin II	Stimulatory	17
• Calcitonin	Stimulatory, then inhibitory	15
• TCP11	Stimulatory, then inhibitory	11
G proteins		
• G alpha s	Stimulatory	23
• G alpha olf	Stimulatory	23
• G alpha i2	Inhibitory	23
mACs		
• mAC3		28
• mAC8		28
• mAC4 (acrosome only)		28
PDEs		
• PDE 4D		36
• PDE 10A		36
• PDE 11A (acrosome only)		36
Decapacitation factor receptor		46

the presence of DF-R in the acrosomal cap and flagellar regions, as well as the postacrosomal region where Ca^{2+}-ATPase is primarily located (43). In addition to showing that the fluorescent signal obtained with the anti-PEBP 1 antiserum is capacitation state-dependent and reversible, we have also shown that addition of DF to capacitated spermatozoa causes conformational and functional changes in adenosine receptors (GPCRs): inactive A_{2A} stimulatory receptors are re-activated and active A_1 inhibitory receptors are inactivated within 5 minutes of DF addition (7,10;).

Based on this new study, we have proposed that mouse sperm DF-R/PEBP 1 plays a fundamental role in sperm physiology by causing alterations in the sperm plasma membrane in both the head and the flagellum. In recent years, investigations of membrane lipid architecture have revealed that the lipids are frequently grouped into distinct domains, commonly referred to as lipid rafts, and many of the proteins located in rafts are known to mediate signal transduction. Interestingly, studies in mammalian spermatozoa have shown that reorganization of marker proteins for sperm surface lipid rafts occurs during capacitation *in vitro* (47,48). Because GPI-anchored proteins are often associated with these rafts (49), it is plausible that DF↔DF-R interactions could contribute to raft dynamics, initiating changes in the plasma membrane that alter the functionality of membrane-associated proteins, including several GPCRs and Ca^{2+}-ATPase. Those proteins, as well as some G proteins and mACs, are located in the same regions as DF-R (29; Table 2), making it feasible for DF-R to act in this way.

8. CONCLUSION AND FUTURE DEVELOPMENTS

When the first review of FPP in *Frontiers in Biosciences* was published (1), we provided a rather simple schematic diagram of how FPP might be able to regulate the production of cAMP. Subsequently, we have found

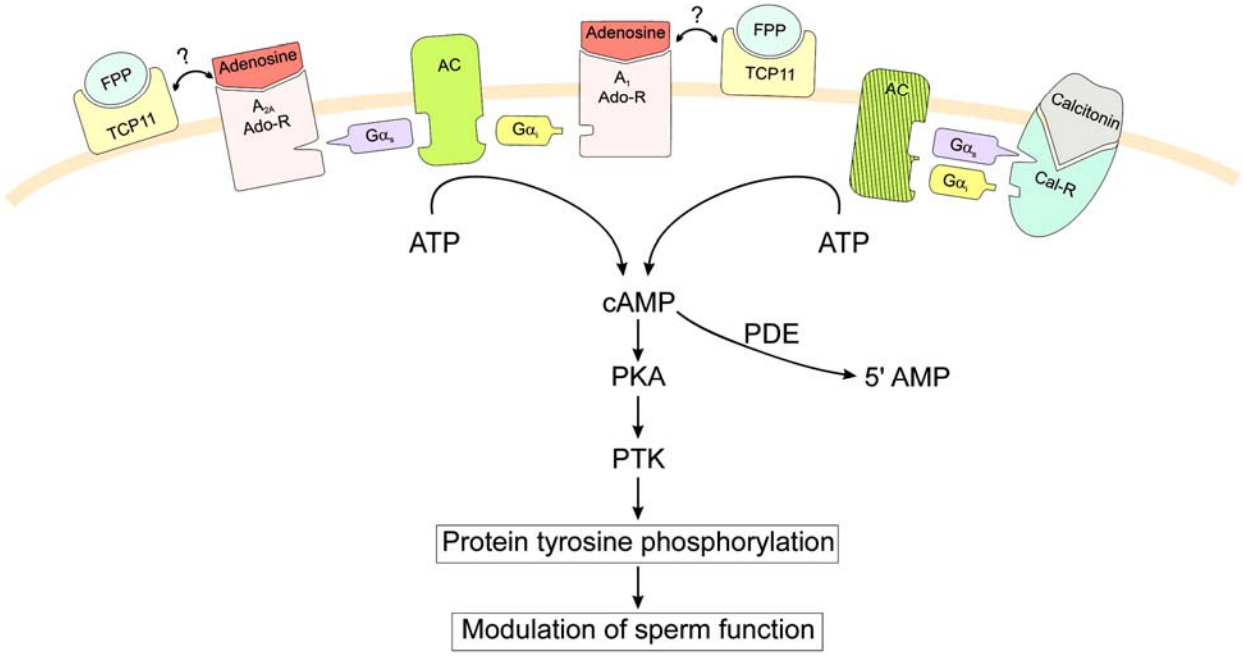

Figure 3. A schematic diagram depicting first messengers interacting with their specific receptors and so regulating mAC activity and cAMP production in a G protein-mediated manner. In uncapacitated spermatozoa, the binding sites on stimulatory A_{2A} adenosine receptors are available; the binding of adenosine to these receptors or of FPP to its receptor, TCP11, will activate the receptors, allowing them to interact with stimulatory G proteins that then activate an mAC isoform to increase production of cAMP. As capacitation proceeds, the A_{2A} receptors change conformation and become inactive, while the inhibitory A_1 adenosine receptors are activated and can inhibit mAC activity via inhibitory G proteins. Only one class of calcitonin receptors has been identified, but in uncapacitated cells they appear to interact with stimulatory G proteins to stimulate AC/cAMP and then, in capacitated cells, to interact with inhibitory G proteins to inhibit AC/cAMP. The consequence of this cAMP inhibition is inhibition of spontaneous acrosome loss but a spermatozoon can still undergo an induced acrosome reaction if it contacts an unfertilized oocyte. Experimental evidence suggests that adenosine and calcitonin receptors regulate AC isoforms that differ in their Ca^{2+} requirement, hence the different patterns on the two ACs depicted. Very recent studies have found that these first messengers can also affect cAMP availability by altering PDE activity.

that several other small molecules, also found in seminal plasma, can regulate cAMP in biologically important ways; the inhibition of the spontaneous acrosome reaction in capacitated spermatozoa is especially significant because this can provide a mechanism for maintenance of sperm fertilizing potential. All these molecules function as first messengers, interacting with GPCRs that then activate/inhibit mAC isoforms. The existence of several different signal transduction pathways, all capable of affecting the production of cAMP, confirms that cAMP plays a pivotal role in the acquisition of fertilizing ability. An expanded schematic diagram of how these first messengers act, based on current evidence, is shown in Figure 3. The complexities revealed by the various studies cited above were unexpected, but make sense if one bears in mind that unregulated cAMP production appears to lead to spontaneous acrosome reactions and consequent loss of fertilizing potential.

By understanding the mechanisms that modulate mammalian sperm function, it may be possible to identify problems that could cause subfertility/infertility and to develop ways to circumvent the problems. For example, we found that some semen samples from men attending infertility clinics had spermatozoa at advanced stages of capacitation prior to incubation under capacitating conditions; this might reflect reduced presence of DF or faulty regulation of mAC/cAMP. Incubation of these samples with a combination of FPP, calcitonin and AII was able to inhibit spontaneous acrosome loss (14). Other possible problems could involve a failure to lose DF and so a failure to under go capacitation and acquire fertilizing ability. Conversely, it should be possible to produce modified DFs that would bind irreversibly to DF-R and so act as a contraceptive, blocking capacitation and so deliberately blocking fertilization.

10. ACKNOWLEDGEMENTS

Recent original research discussed in this review has been supported by grants to LRF from several sources, including The Wellcome Trust, the Kinetique Biomedical Seed Fund, the Biotechnology and Biological Sciences Research Council (BBSRC) and Pfizer Global Research and Development (Sandwich, UK). Rachel Gibbons is the recipient of a CASE-BBSRC PhD studentship, with Pfizer Global Research and Development being the industrial partner for the award.

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Key words: Egg, Ovum, Capacitation, Fertilization, Sperm, Spermatozoa, Decapacitation Factor, GPCR, G proteins, mAC, PDE, PEBP 1, Review

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