CHAPTER 4

Discussion
Fertilization in mammals represents the beginning of life for a new individual and it is preceded by a number of events, which occur in an obligatory sequence starting with gametogenesis and culminating with the fusion of the gametes, the spermatozoon and the oocyte. Spermatozoa are equipped with a limited repertoire of modifications that exclusively serve their purpose to fertilize the oocyte. When produced in testis, spermatozoa are immotile; they acquire the ability to move progressively as they transit through the epididymis. However, complete fertilization-competence of spermatozoa is acquired only in the female reproductive tract by a process called capacitation. Capacitation refers to all the molecular and physiological events associated with the spermatozoa in the female reproductive tract which make them fertilization-competent. This maturational event can be achieved in in vitro in a defined medium and it is known to be correlated with other changes such as hyperactivation (change in motility) and acrosome reaction. In the present study, attempts have been made to understand the molecular basis of this important but poorly understood phenomenon of capacitation using golden hamster (Mesocricetus auratus) as the model system.

4.1 Computer-aided Sperm Analysis of Hamster Spermatozoa

A computer-aided motility analyzer (HTM-IVOS) was used to monitor the motility pattern and motility parameters of hamster spermatozoa. The conditions and the parameter set up values for the motility analysis were as described earlier and were validated as recommended by the manufacturers and by Yeung et al (1992). Earlier studies have indicated that hamster spermatozoa under appropriate conditions conducive for capacitation exhibit a distinct change in their motility, termed
hyperactivation (Yanagimachi, 1969a, b, 1970) characterised by the transformation of the linear progressive type of motility to a non-progressive type of motility with whiplash-like beatings of the tail resulting in spermatozoa that exhibit various motility patterns such as 'circular', 'helical' and 'figure of eight' (Gwatkin and Anderson, 1969; Yanagimachi, 1969a, b, 1970; Suarez et al., 1984, 1986; Drobnis et al., 1988; Suarez, 1988; Aoki et al., 1994; Shivaji et al., 1995; Jayaprakash et al., 1997; Ain et al., 1999) (Fig. 1, Chapter 1). Further, it was also observed that, in hamster, hyperactivation of the spermatozoa is normally seen after 1-2 h of incubation reaching a maximum by 4 h (Llanos and Meizel, 1983; Suarez et al., 1984 Cherr et al., 1986; Cummins and Yanagimachi, 1986; Suarez, 1988). The present study confirms the above earlier observations that hyperactivation of hamster spermatozoa occurs by 1-2 h of incubation and reaches a maximum by 4-5 h (Fig. 2, Chapter 3). In addition, the study also confirms that the hyperactivated spermatozoa are predominantly of two types, the "circular" and "helical". However, "the figure-of-eight" pattern was not consistently observed by all and when seen they constituted < 5% of all the capacitated spermatozoa (Yanagimachi, 1970; Suarez, 1988).

Motility parameters of spermatozoa such as VSL, VAP, VCL, STR, LIN, BCF and ALH (Table 1, Chapter 2) provide valuable information on the acquisition of progressive motility in spermatozoa during epididymal maturation and the development of hyperactivation during capacitation. In fact, in the current investigation the two classes of hyperactivated spermatozoa (the circular and the helical) could be differentiated from one another and the non-hyperactivated spermatozoa exhibiting planar motility based on the distinct differences in VSL, VCL, and ALH (Table 2, Chapter 1). Linearity,
progressive velocity, and amplitude of lateral head displacement of a spermatozoon have been used earlier to differentiate and sort hyperactivated human spermatozoa from non-hyperactivated spermatozoa (Burkman, 1991). Normally, hyperactivated spermatozoa are less progressive and thus, have a low VSL, STR and additionally exhibit increased ALH. In the present study the circular moving spermatozoa were the only spermatozoa that exhibited significant reduction in VSL, LIN, and STR and increase in ALH compared to the non-hyperactivated spermatozoa, thus confirming the definition of hyperactivation. The hyperactivated spermatozoa in hamster and human are similar in that, they have high VCL, low VSL, low LIN (Morales et al., 1988), and high ALH (Tesarik et al., 1990). Similar changes with respect to increased ALH and reduction in progressive motility have been observed during hyperactivation of spermatozoa from guinea pigs (Katz et al., 1978; Katz and Yanagimachi, 1981), monkeys (Behboodi et al., 1987), rabbits (Johnson et al., 1981; Suarez et al., 1983) and rams (Cummins, 1982). Increased ALH in hyperactivated spermatozoa is a significant functional change and it has been correlated with the fertilizing ability of human spermatozoa (Aitken et al., 1985; Jeulin et al., 1986; Mortimer et al., 1986).

Hyperactivation of spermatozoa may be absolutely essential in vivo, for the spermatozoa to successfully encounter the various barriers prior to binding and fusing with the oocyte. Hyperactivated motility patterns in vivo were observed as 'figure of eight' and 'darting' in the oocyte-cumulus complexes (Corselli and Talbot, 1986), the hatchet type in the cumulus matrix (Drobnis et al., 1988) and spermatozoa that beat their tails with high beat cross frequency and acute bending as in the zona pellucida (Katz et al., 1986). Further, there is evidence to indicate that hyperactivation is
beneficial to spermatozoa for detaching from the oviductal mucosa, for passing through the viscoelastic substances in the oviduct and for penetrating the zona pellucida (Suarez, 1996).

4.2 Effects of Media Components on Motility and Hyperactivation of Hamster Spermatozoa

Very little is known about the biochemical pathways regulating hyperactivation of spermatozoa. However, it has been demonstrated that extracellular Ca$^{2+}$ is required for hyperactivation in vitro (Yanagimachi, 1982; Fraser, 1987). Consistent with these findings it was observed that in hamster spermatozoa the absence of extracellular Ca$^{2+}$ inhibited motility severely, the tail was bent and therefore, hyperactivation as anticipated was not observed (Fig. 2A, Chapter 3). The requirement for extracellular calcium for hyperactivation appears to be related to cAMP availability. Further, studies have indicated that cAMP acts downstream to calcium influx during hyperactivation, because the inhibitory effect on hyperactivation due to the absence of calcium could be reversed by adding cell-permeable cAMP analogues (Aoki et al., 1999).

As capacitation and hyperactivation occur concomitantly the effects of other media components namely BSA and NaHCO$_3$ which are known to influence capacitation were also studied. Hamster spermatozoa incubated in TALP showed a time-dependent increase in hyperactivation in vitro (Fig. 2, Chapter 3). However, when the spermatozoa were incubated in a medium lacking both BSA and PVA the percentage motility was lowered very significantly and no hyperactivation was observed (Fig. 2A, Chapter 3). This indicated that BSA and/or PVA are essential for the hyperactivated motility in
hamster spermatozoa. Further, it was observed that hyperactivation was normal when either of these two components that is BSA or PVA was present in the medium suggesting that BSA can be replaced by PVA in the medium. This is consistent with the observations that the presence of either of the two in TALP was sufficient for acrosome reaction and protein tyrosine phosphorylation (as described later). A similar observation in hamster spermatozoa was reported earlier (Uto and Yamahama, 1996) which suggested that PVA could substitute BSA in the capacitation medium.

The effect of NaHCO₃ on hyperactivation was also studied as this is also known to regulate hyperactivation (Boatman and Robbins, 1991; Shi and Roldan, 1995a, Neill and Olds-Clarke, 1987). It was observed that when hamster caudal spermatozoa were incubated in a medium, that lacked sodium bicarbonate, but contained HEPES buffer to maintain the pH the hyperactivation was delayed up to 3 h; but by 5 h the percentage hyperactivation reached the control level. There is some discrepancy in the present study and the results of Boatman and Robbins (1991) who demonstrated that in NaHCO₃ depleted TALP hamster spermatozoa were not hyperactivated. However, recently Visconti et al (1999c) also demonstrated that in hamster spermatozoa the capacitation-associated protein tyrosine phosphorylation was delayed in the absence of NaHCO₃ but not totally inhibited. Therefore, the present results and the results of Visconti et al (1999c) would imply that NaHCO₃ is not absolutely essential for hyperactivation or other downstream events of capacitation.
4.3 Effects of Media Components on Acrosome Reaction of Hamster Spermatozoa

The requirement of BSA for the induction of acrosome reaction was shown in hamster (Lui and Meizel, 1977), mouse (Fraser, 1985) human (Suarez et al., 1986) and other species. It appears that albumin induces acrosome reaction by causing cholesterol efflux from the sperm plasma membrane. This is supported by the demonstration that cyclodextrin, a specific cholesterol binding agent can replace BSA from the medium and thus can induce acrosome reaction in the absence of BSA, as shown in goat (Ibarra et al., 2000), human (Cross, 1999) and mouse (Visconti et al., 1999b) spermatozoa. Furthermore, cyclodextrin has been shown to induce acrosome reaction in post-thaw boar spermatozoa (Zeng and Terada, 2000). In the present study also, it was observed that the percentage of hamster spermatozoa undergoing spontaneous acrosome reaction increases in a time-dependent fashion when the medium contains either BSA or PVA (Fig. 3, Chapter 3). This suggests that PVA like BSA probably acts as cholesterol scavenging agent and therefore, in the absence of BSA (but in presence of PVA) there was no inhibition in the percentage of spermatozoa undergoing the acrosome reaction. However, when both these components were absent from the medium the percentage motility dropped down very significantly and therefore, scoring percentage acrosome reaction became extremely difficult as most of the immotile spermatozoa tend to undergo degenerate acrosome reaction.

The role of NaHCO₃ in the induction of acrosome reaction has been investigated in the spermatozoa of hamster (Boatman and Robbins, 1991; Visconti et al., 1999c), mouse (Lee and Storey, 1986; Shi and Roldan, 1995a), guinea pig (Bhattacharya and
Yanagimachi, 1988) and bull (Spira and Breitbart, 1992). In hamster spermatozoa, NaHCO₃ was found to be absolutely essential for the acrosome reaction and it was also observed that the effect was not due to pH because even when the pH was maintained, the absence of NaHCO₃ inhibited acrosome reaction (Boatman and Robbins, 1991). Further, they found that bicarbonate was required for the initial stages of capacitation because if the concentration of NaHCO₃ was decreased in the medium to suboptimal level after 5 h, the decrease in acrosome reaction was not significant. This was further supported by Visconti et al. (1999c) who demonstrated that NaHCO₃ was essential for spontaneous acrosome reaction in hamster spermatozoa since in the absence of it, acrosome reaction did not increase above the basal level (<10%) even up to 8 h of incubation whereas in the complete medium (contains 25 mM NaHCO₃) the acrosome reaction reached the maximum level (~ 90%) in 4-5 h. It was also demonstrated that specific anion-channel blockers namely DIDS and SITS which prevent the entry of NaHCO₃ into cells, inhibited the percentage acrosome reaction (Visconti et al., 1999c). Surprisingly, in contrast to these earlier studies, in the present study it was observed that if the pH of the medium was maintained in the absence of NaHCO₃ by HEPES the acrosome reaction of hamster spermatozoa was not inhibited and the spermatozoa incubated in such a medium underwent acrosome reaction just as in the control. NaHCO₃ was shown to be essential for acrosome reaction in mouse (Lee and Storey, 1986), and bull (Spira and Breitbart, 1992). But, in contrast, Shi and Roldan (1995a) demonstrated that NaHCO₃ was absolutely required for capacitation (as studied by CTC fluorescence) but not for acrosomal excocytosis. In fact, earlier studies also indicated that NaHCO₃ was not required for acrosome reaction in goat (Kusunoki et al., 1989) and
guinea pig spermatozoa (Bhattacharya and Yanagimachi, 1988).

The mechanism by which NaHCO₃ influences acrosome reaction is probably mediated through the cAMP dependent PKA pathway involving protein tyrosine phosphorylation (Spira and Breitbart, 1992; Visconti et al., 1999a, b). At the moment, the reasons for the contradictory observations of the effect of NaHCO₃ on hamster acrosome reaction are yet to be sorted out. Nevertheless, the discrepancy could be attributed to species-specificity, or in part, to the way the acrosome reaction has been scored by different investigators.

It is a well established fact that Ca²⁺ is absolutely essential for acrosome reaction of spermatozoa in all mammalian species (Yanagimachi, 1994; Suarez and Dai, 1995). In the present study also the percentage motility, hyperactivation and acrosome reaction were severely impaired in the absence of Ca²⁺.

4.4 Protein Tyrosine Phosphorylation during Capacitation

Spermatozoa from a variety of mammals when capacitated in vitro, exhibit a time-dependent increase in protein tyrosine phosphorylation (Visconti et al., 1995a, b; 1999c; Leclerc et al., 1996; Galantino-Homer et al., 1997; Carrera et al., 1996; Kalab et al., 1998). The present study demonstrates that hamster spermatozoa also exhibit a time dependent increase in protein tyrosine phosphorylation (Fig. 4, Chapter 3) and the increase correlated with sperm capacitation, as judged by the acrosome reaction (Fig. 3, Chapter 3), thus confirming the recent observations of Visconti et al (1999c).

Studies on the effect of NaHCO₃, Ca²⁺, BSA and PVA on protein tyrosine phosphorylation clearly highlighted that regulation of protein tyrosine phosphorylation
during capacitation is species-dependent. For instance, the requirement for NaHCO$_3$ by hamster spermatozoa was not mandatory, since the absence of NaHCO$_3$ caused a delay in phosphorylation up to 1 h but recovered to control levels by 3 h (present results and Visconti et al., 1999c). But, earlier studies have indicated that NaHCO$_3$ was required by mouse (Visconti et al., 1995a; Shi and Roldan, 1995a) and human spermatozoa (Luconi et al., 1996; Carrera et al., 1996). The present study also demonstrates that the requirement of Ca$^{2+}$ for the capacitation-associated increase in protein tyrosine phosphorylation was also not obligatory and by 3 h of capacitation phosphorylation of proteins was as in the control spermatozoa. Human spermatozoa also do not require Ca$^{2+}$ (Luconi et al., 1996; Carrera et al., 1996) but Ca$^{2+}$ was required by mouse spermatozoa (Visconti et al., 1995a). Visconti et al (1998) proposed a working model for regulation of sperm capacitation in which they proposed that both HCO$_3^-$ and Ca$^{2+}$ exert their effects through adenylyl cyclase. If this is so, then the absence of either of the effectors (HCO$_3^-$ or Ca$^{2+}$) may not affect the capacitation process. In fact, this may be the case in hamster spermatozoa since absence of Ca$^{2+}$ or NaHCO$_3$ did not effect capacitation. But absence of both decreased the capacitation-associated protein tyrosine phosphorylation probably due to a decrease in the activity of adenylyl cyclase (which requires either Ca$^{2+}$ or NaHCO$_3$) thus leading to a decrease in cAMP which is required for protein kinase A, which in turn regulates protein tyrosine kinases (Visconti et al., 1998). Thus, it appears that the signal transduction pathway with respect to the influence of Ca$^{2+}$ and NaHCO$_3$ on capacitation is not identical in mouse, human and hamster spermatozoa (Table 1). Earlier studies have also indicated that tyrosine phosphorylation in human spermatozoa could be independent of Ca$^{2+}$
(Mendoza et al., 1995) and both tyrosine kinase-dependent and independent processes are involved in Ca$^{2+}$ uptake (Tesarik et al., 1996).

In this study it was also observed that protein tyrosine phosphorylation of hamster spermatozoa during capacitation was not affected by the absence of either BSA or PVA. The reason for this could be the ability of these two constituents (viz. BSA and PVA) to substitute for the absence of the other and facilitate capacitation (Uto and Yamahama, 1996; Bavister, 1981). However, in the absence of both BSA and PVA there was a significant decrease in protein tyrosine phosphorylation, thus confirming earlier observations that BSA was absolutely essential for the capacitation-associated increase in protein tyrosine phosphorylation of mouse and human spermatozoa (Visconti et al., 1995a; Luconi et al., 1996; Carrera et al., 1996). It has also been demonstrated that BSA influences capacitation by acting as a sink for cholesterol. Removal of cholesterol positively influences adenylyl cyclase (Visconti et al., 1998; 1999b).
Table 1. Influence of Ca\textsuperscript{2+}, NaHCO\textsubscript{3} and BSA on the capacitation-associated protein tyrosine phosphorylation, hyperactivation and acrosome reaction of mammalian spermatozoa\textsuperscript{a}

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\textsuperscript{a}The data represent only those studies in which attempts were made to correlate protein tyrosine phosphorylation with hyperactivation and (or) acrosome reaction. \textsuperscript{b}R, NR and - indicate required, not required and data not available, respectively. \textsuperscript{c}Indicates data from the present study.
4.4.1 Is Hyperactivation Linked to Protein Tyrosine Phosphorylation?

Fertilization still represents the benchmark endpoint of capacitated spermatozoa and is preceded by hyperactivation and acrosome reaction. The present study confirms our earlier observations and those by others that hyperactivation of spermatozoa in hamster is normally seen 1-2 h after incubation under conditions that support capacitation (Suarez, 1988; Shivaji et al., 1995) and reaches a peak after 5 h. This temporal sequence of increase in percentage hyperactivation of hamster spermatozoa from 1 to 5 h coincides with the time course of protein tyrosine phosphorylation, thus implying that hyperactivation and protein tyrosine phosphorylation may be related or linked. If these two processes are linked then factors such as Ca$^{2+}$, BSA and NaHCO$_3$ which may effect any one of the processes (i.e., hyperactivation / acrosome reaction or protein tyrosine phosphorylation) should obviously affect the other. With this in view, the effects of depleting the medium of Ca$^{2+}$ BSA and NaHCO$_3$ were studied on the above two processes. The present study demonstrates that hamster spermatozoa incubated in TALP minus NaHCO$_3$ or Ca$^{2+}$ or BSA and PVA showed a decrease in protein tyrosine phosphorylation and simultaneously a delay in hyperactivation (as in the absence of NaHCO$_3$) or inhibition of hyperactivation (as in the absence of Ca$^{2+}$ or BSA and PVA) thus indicating a possible correlation between decrease in protein tyrosine phosphorylation and delay or inhibition in hyperactivation. The present results confirm that hamster spermatozoa require Ca$^{2+}$ for hyperactivation (White and Aitken, 1989) and do not require BSA if PVA is present in the medium (Uto and Yamahama, 1996). However, we were unable to confirm the results of Boatman and Robbins (1991) who demonstrated that in NaHCO$_3$ depleted TALP hamster spermatozoa were not hyperactivated. At the moment, we have no explanation for this discrepancy but we
would like to add that the capacitation-associated protein tyrosine phosphorylation was delayed in the absence of NaHCO₃, thus confirming the results of Visconti et al (1999c).

4.4.2 *Is Acrosome Reaction Linked to Protein Tyrosine Phosphorylation?*

It has also been established that hamster spermatozoa do not require BSA for acrosome reaction if the medium contains PVA (Uto & Yamahama, 1996) as observed in the present study. However, NaHCO₃ was absolutely essential for acrosome reaction, even when the pH of the medium was maintained (Boatman & Robbins, 1991; Visconti et al., 1999c). In the present study, however, it was observed that neither the motility nor the percent acrosome reaction was affected in the absence of NaHCO₃. But, such a medium contained 25 mM HEPES. Further, it has also been demonstrated that Ca²⁺ is essential for the acrosome reaction of all mammalian spermatozoa (Yanagimachi, 1994; Suarez & Dai, 1995). The question we would like to ask is whether acrosome reaction is linked to protein tyrosine phosphorylation. This is not probably the case because the kinetics of protein tyrosine phosphorylation appears to be faster in comparison to the kinetics of acrosome reaction (Visconti et al., 1999c and the present study). That the two processes are not linked is further supported by the observation that Ca²⁺ which is absolutely essential for acrosome reaction only delayed the protein tyrosine phosphorylation of hamster spermatozoa incubated under capacitation conditions.

4.5 *Role of PTK and PTP in Capacitation of Hamster Spermatozoa*

The present results clearly indicate the involvement of protein tyrosine kinase(s) and phosphatase(s) in the regulation of sperm capacitation since an increase in protein tyrosine phosphorylation was observed during capacitation of hamster spermatozoa.
This increase in protein tyrosine phosphorylation could either be due to the stimulation of a tyrosine kinase, or due to the inhibition of a protein tyrosine phosphatase, or both. Thus, an understanding of the kinase(s) and the phosphatase(s) involved would definitely help in understanding the mechanism underlying capacitation. With this in view, specific inhibitors of PTK and PTP were used to study their effects on motility, hyperactivation and capacitation.

It was observed that neither genistein (4', 5, 7-Trihydroxy isoflavone), a competitive inhibitor of ATP binding to the tyrosine kinase, (Akiyama et al., 1987) nor tyrphostin (Novogrodsky et al., 1994), a competitive inhibitor of substrate binding to the kinase did not inhibit the motility of intact mature hamster spermatozoa but piceatannol (3, 3', 4, 5' - Tetrahydroxy-transstibene), another PTK inhibitor (Geahlen and McLaughlin, 1989) caused a time- and a dose-dependent decrease in the percentage of motile spermatozoa. Piceatannol, also brought about a qualitative change in the motility of hamster spermatozoa as evidenced from the fact that the treated spermatozoa exhibited slower motility, due to a time- and a dose- dependent decrease in the velocity parameters and ALH. But, the frequency of flagellar beating remained unaltered except for a sudden increase at 90 min in the presence of 300 μM piceatannol when less than 5% of spermatozoa were motile but were very sluggish. The STR and LIN of trajectories of the spermatozoa were higher in the presence of piceatannol compared to control where it fluctuated along a narrow range. It is extremely difficult to attribute a reason for the increase in STR and LIN since these two parameters are derived from the velocity parameters VAP, VSL and VCL, which were also affected. The reason, why genistein and tyrphostin were ineffective whereas piceatannol was very effective is not clear. In a
recent study, Pukazhenthi et al (1998) also observed that genistein and tyrphostin had no effect on the motility of cat spermatozoa. Further, Ashizawa et al (1998) demonstrated that methyl 2,5-dihydroxycinnamate (2,5-Mec), a specific inhibitor of protein tyrosine kinase and an inhibitor more permeable to the plasma membrane inhibited the motility of fowl spermatozoa. Therefore, the inability of genistein and tyrphostin to inhibit motility may be due to the fact that they are less permeable, or less effective than piceatannol.

Sodium orthovanadate is an inhibitor of protein tyrosine phosphatase. However, it is known to inhibit some other enzymes such as ATPase, and alkaline phosphatase. Nevertheless, it has been used to implicate the role of PTP in various cell functions (Gordon, 1991). In the present study, sodium orthovanadate was found to inhibit the motility of spermatozoa in a time- and a dose- dependent manner. In the presence of sodium orthovanadate the velocity parameters such as VAP, VSL, and VCL were significantly lower in values when compared to the control, however, STR, LIN and BCF were not affected. By 30 min there was a drastic drop in the percent motility and it decreased to 20%. This dose- and time-dependent affect of sodium orthovanadate on motility indicates that PTP activity is needed for motility. However, the observed effect on motility through the effect of sodium orthovanadate on other target enzymes cannot be ruled out. For this purpose a specific PTP inhibitor would be required. To the best of our knowledge such an inhibitor is not known.

Relatively little is known about the role of protein phosphatases in sperm motility (Tash and Bracho, 1994). In dog and human spermatozoa it has been proposed that PP-2B, a Ca²⁺calmodulin-dependent protein phosphatase, was probably involved in
modulating sperm motility (Tash et al., 1988; Ahmad et al., 1995). More recently, it was demonstrated that a functional PP-1 system was present in bovine, human, and rhesus monkey spermatozoa and calyculin and okadaic acid, specific inhibitors of PP-1 induce motility in caput spermatozoa and stimulate the kinetic activity of mature cauda epididymal spermatozoa. Based on these observations it was postulated that protein phosphatase 1 is a biochemical mediator of motility (Smith et al., 1996) in mammalian spermatozoa. Ashizawa et al. (1997) also demonstrated that PP-1 mediated dephosphorylation of proteins of the axoneme and/or cytoskeletal components of fowl spermatozoa may be involved in the inhibition of motility. Thus, these recent studies seem to indicate a role for a cytosolic PP-1, a serine/threonine phosphatase, in sperm motility. Further, it also appears that certain phosphatases like PP-1 are negative regulators of sperm motility (Smith et al., 1996; Vijayraghavan et al., 1996; Ashizawa et al., 1997). Others may be positive regulators like the PTP in hamster spermatozoa which when inhibited leads to a decrease in sperm motility (Uma Devi et al., 1999). Hyperactivation was not observed in the presence of these PTK and PTP inhibitors thus implying the involvement of these enzymes in hyperactivation and/or capacitation. If this is so then inhibition of these enzymes should also be reflected at the level of protein tyrosine phosphorylation of the effector proteins during capacitation. In fact, it was observed that genistein and tyrphostin did not affect the capacitation-associated protein tyrosine phosphorylation of hamster spermatozoa whereas piceatannol decreased the phosphorylation very drastically. On the other hand sodium orthovanadate increased the tyrosine phosphorylation of the 83 kDa protein when compared to the control. It should be noted (as described later also) that the 83 kDa protein is the major tyrosine
phosphorylated protein during capacitation. The increase in protein tyrosine phosphorylation in the presence of sodium orthovanadate was very much expected. Thus, it is obvious that both PTP and PTK activity are essential for capacitation and both these enzymes together regulate capacitation. However, one may argue here that sodium orthovanadate should have a positive effect on motility like it has on capacitation, but it should be noted that by inhibiting PTP activity the equilibrium of phosphorylation is disturbed which in turn may have a negative impact on motility. It can be emphasized here that protein tyrosine phosphorylation is a molecular event while motility is a physiological function.

So far only two PTK have been purified from mature spermatozoa. sp42, a PTK from boar (Berruti & Porzio, 1992; Berruti, 1994) and a 45 kDa plasma-membrane associated PTK from hamster spermatozoa (Uma Devi et al., 2000). The sp42 from boar is germ cell specific but it is not known if it is plasma membrane-associated or cytosolic in nature. The PTK from hamster spermatozoa differs from sp42 in that it has a mol. wt. of 45 kDa and it is plasma membrane-associated. Similarly there are ample evidences for the presence of PTP in spermatozoa but none of them have been purified and characterized. Further studies on these kinases and phosphatases will be very helpful to understand the mechanism underlying capacitation.

4.6 Molecular Basis of Sperm Capacitation

The present study confirms that hamster cauda epididymal spermatozoa following incubation in a medium conducive for capacitation exhibit a capacitation-dependent increase in tyrosine phosphorylation of a subset of proteins in the range of
50-144 kDa (Visconti et al., 1999c). Similar studies with spermatozoa of mouse (Visconti et al., 1995a), bovine (Galantino-Homer et al., 1997) and human (Carrera et al., 1996; Luconi et al., 1996; Leclerc et al., 1996) had also established a correlation between increase in protein tyrosine phosphorylation and capacitation. Thus, the correlation between capacitation and the protein tyrosine phosphorylation appears to be a general phenomenon in the species studied so far. These studies suggest that protein tyrosine phosphorylation can ultimately be important in controlling events leading either to the capacitated state or to the events occurring as a consequence of capacitation since cause- and effect- relationship between protein tyrosine phosphorylation and capacitation is not understood. The observed increase in protein tyrosine phosphorylation during capacitation follows relatively a slower kinetics when compared to tyrosine phosphorylation occurring in other somatic cells (Sefton and Campbell, 1991; Iwashita and Kobayashi, 1992). In this context, it can be emphasized that the increase in protein tyrosine phosphorylation during capacitation occurs in the absence of an apparent external stimulus unlike the somatic cells where the same is regulated by extrinsic factors like growth factors and other such ligands (Iwashita and Kobayashi, 1992). Thus, it is apparent that signaling cascade is triggered intrinsically in spermatozoa during capacitation, however, extracellular environment does affect the signaling event occurring during capacitation. Therefore, it is very interesting to study how mammalian spermatozoa might intrinsically control events that lead to capacitation. One likely set of controlling factors related to this maturational event lies within the plasma membrane because capacitation has been shown to be accompanied by changes in membrane composition and structure, as assessed by
changes in the distribution of a variety of surface proteins (Yanagimachi, 1994), changes in the distribution of intramembranous particles (Koehler and Gaddum-Rosse, 1975; Suzuki and Yanagimachi, 1989) and a reduction in the density of filipin-sterol complexes (Suzuki and Yanagimachi, 1989). Such changes could influence membrane fluidity (Wolf and Cardullo, 1991) which may act as the trigger for the signaling cascade.

4.7 Identification of the Prominent Phosphotyrosine Protein of 83 kDa in Hamster Capacitated Spermatozoa as AKAP

The present study and earlier studies on spermatozoa of hamster (Visconti et al., 1999c), mouse (Visconti et al., 1995a), bovine (Galantino-Homer et al., 1997) and human (Carrera et al., 1996; Luconi et al., 1996; Leclerc et al., 1996) establish a correlation between increase in protein tyrosine phosphorylation and capacitation. Therefore, there is a need to identify and characterize these phosphotyrosine proteins so as to understand their role in capacitation. The predominant phosphotyrosine protein of capacitated hamster spermatozoa of 83 kDa was identified as an homologue of AKAP82 of bovine, human, mouse and rat spermatozoa (Carrera et al., 1996; Johnson et al., 1997; Brito et al., 1989; Moss et al., 1999; Vijayaraghavan et al., 1997; Vijayraghavan et al., 1999) based on its mol. wt., pI, amino acid sequence and localization. The mol. wt. of 83 kDa observed for the AKAP of hamster spermatozoa is similar to that reported for the AKAP82 of mouse (82 kDa), bovine (84 kDa), human (82 kDa) and rat (80 kDa) spermatozoa (Turner et al., 1998). Further, using antibodies to hamster AKAP83 it was observed that the protein exists in two forms namely as pro-
AKAP83 (mol. wt. 97 kDa) which by proteolytic cleavage yields AKAP83 (mol. wt. 83 kDa). This observation is consistent with the existence of pro-AKAP82 homologues in mouse (Carrera et al., 1994) human (Turner et al., 1998) and bovine spermatozoa (Moss et al., 1999). From the immunoblot analysis of capacitated hamster spermatozoa using anti-AKAP83, it is obvious that a major part of pro-AKAP83 is proteolytically processed and only a small fraction still exists as pro-AKAP83. The processing in hamster spermatozoa appears to be similar to that observed in the case of mouse and bovine spermatozoa (Carrera et al., 1994; Moss et al., 1999) but differs from that observed in human spermatozoa where pro-hAKAP82 is present in larger amount relative to hAKAP82 implying that proteolytic processing was not as complete (Carrera et al., 1996).

Sequence analysis of cDNA clones or RT-PCR products of pro-AKAPs from mouse (Carrera et al., 1994), bovine (Moss et al., 1999), human (Turner et al., 1998) and hamster (present study), indicate that the pro-AKAP82 homologues are highly conserved. This is further confirmed by the high homology (>85%) observed between pro-AKAP83 and pro-AKAP82 homologues at the amino acid level. Interestingly the hamster pro-AKAP82 exhibited 91% identity with mouse and rat pro-AKAP82 indicating that it is more closely related to the rodents than to the bovine and human species (the higher mammals) with which the identity was 81 and 78% respectively. The fact, that pro-AKAP82 homologues including pro-AKAP83 of hamster are functionally also conserved is clear from the presence of specific domains like the RII binding domain which binds specifically to the RII regulatory subunit of PKA (Mandal et al., 1999) and the pro-AKAP82/AKAP82 proteolytic cleavage site (Moss et al., 1999;
Carrera et al., 1994; Turner et al., 1998). These two domains are highly conserved and identical in all the homologues of AKAP82 except in bovine AKAP110 (Vijayaraghavan et al., 1997) that lacked the pro-AKAP82/AKAP82 cleavage site. In addition, in bovine AKAP110, the RII binding domain was similar but not identical to homologues of AKAP82 but this domain was predicted to form an amphipathic helix, a characteristic feature of this domain in all AKAPs. Though the ability to bind the RII subunit of PKA has been used to identify AKAPs, its presence as a conserved domain with total identity at the sequence level would also imply a similar function for this domain in hamster. In fact, it would be all the more important to investigate whether AKAPs function as scaffolding proteins for a variety of proteins such as kinases and phosphatases that are involved in sperm motility or capacitation.

Immunofluorescence studies using anti-AKAP83 antibodies indicated that in hamster spermatozoa AKAP83 is localized to the principal piece of the tail implying that AKAP83 is associated with the fibrous sheath. Similar pattern of localization was also seen in spermatozoa of bull (Moss et al., 1999), mouse (Carrera et al., 1994; Johnson et al., 1997) and human (Turner et al., 1998). Taken together these results would indicate that lack of immunoreactivity with the midpiece and endpiece of the tail would imply the absence of AKAPs in the outer dense fibres, mitochondria, axoneme and plasma membrane. However, in addition to AKAP83 which is confined to the principal piece of the tail, other phosphotyrosine containing proteins may be present not only in this region but also in other regions of the spermatozoa. In fact, using anti-phosphotyrosine antibody proteins could be localized in the principal piece, midpiece and anterior region of the head of hamster spermatozoa (present study), in the
principal piece and neck region (Carrera et al., 1996) and in the acrosomal region (Naz et al., 1991) of human spermatozoa.

4.7.1 Tissue Specific Expression of Pro-AKAP83 and AKAP83

Using a combination of primers capable of detecting pro-AKAP83 and AKAP83 it has been possible to demonstrate that these two proteins are expressed only in the testis and are therefore, testis specific. Absence of RT-PCR products in the other organs suggests that pro-AKAP83 and AKAP83 are absent in the organs tested but does not preclude the possibility that other AKAPs are present in these organs. In mouse AKAP82 message was not detected in brain, liver and spleen but was detected in postmeiotic germ cells namely the round and the condensed spermatids (Carrera et al., 1994). Further, using anti-AKAP82 antibodies, it was demonstrated that AKAP82 was synthesized as a precursor during spermiogenesis and therefore could be detected only as pro-AKAP82 in round and condensed spermatids and the mature AKAP82 was visible as a highly enriched protein in the tail fraction of whole spermatozoa. This observation indicates that pro-AKAP82 and AKAP82 are spermatid and spermatozoa specific. It is also of interest to note that the synthesis of AKAP82 as a precursor during spermiogenesis coincides with the assembly of the fibrous sheath that is present only in the principal piece of the sperm tail. In fact, it has been demonstrated that mAKAP82 is synthesized in the cell body of condensed spermatids as pro-AKAP82 and then transported down the flagellum for assembly into the fibrous sheath of the principal piece (Johnson et al., 1997; Carrera et al., 1994).
4.7.2 Function of AKAP83 in Hamster Spermatozoa

Recent observations, that AKAP82 homologues are conserved in the spermatozoa of various mammalian species, are localized to the fibrous sheath of the principal piece of the tail and bind the type II regulatory subunit of PKA has led to the hypothesis that AKAP82 homologues in spermatozoa regulate signal transduction pathways controlling initiation and sustenance of spermatozoal motility. This could be achieved by AKAP82 anchoring PKA (through its regulatory subunit) to the fibrous sheath such that PKA comes in close proximity to its substrates which it phosphorylates in the presence of cAMP. Interestingly, it has also been demonstrated that cAMP is required for the sliding of the fibrous sheath in demembranated spermatozoa (Si and Okuno, 1993). In addition, synthetic peptides which inhibit PKA binding to AKAP affect the anchoring of PKA to the fibrous sheath and thus inhibit motility of bovine cauda epididymal spermatozoa (Vijayaraghavan et al., 1997). The present study does not provide any direct information on the function of AKAP83 with respect to hamster spermatozoal motility. However, at the same time, it is tempting to speculate that AKAP83 in hamster which has been detected in caput and cauda epididymal spermatozoa influences motility probably in the same way as it does in other spermatozoa, since it shares the important characteristics of being localized in the fibrous sheath and possesses the conserved RII binding domain. However, the role of AKAP in sperm motility because of its ability to act as an anchoring protein of PKA has been questioned since RII ‘knock out’ mice showed no obvious fertility defects (Burton et al., 1997). More recently it was also demonstrated that differences in motility in spermatozoa from normal men was not dependent on the processing or tyrosine phosphorylation of pro-AKAP82/AKAP82 (Turner et al., 1998). Further, in
men with dysplasia of the sperm fibrous sheath, characterized by male infertility, asthenozoospermia and morphologically abnormal flagella, it was observed that AKAP4 (same as AKAP82) and AKAP3 (same as AKAP110) showed no qualitative or quantitative differences in the patients and normal men. Further, these AKAPs also retained their ability to bind RII, were localized to the fibrous sheath and showed no alterations in the gene sequence in the binding domain (Turner et al., 2001). Based on all the available information it is still not clear as to what could be the function of AKAP in the fibrous sheath despite the observation that it could bind to the regulatory subunit of PKA, tether it close to its substrate and thus influences phosphorylation.

Unlike mouse and rat AKAP82 which are phosphorylated at the serine/threonine residues the hamster AKAP83 and human AKAP82 are phosphotyrosine proteins which exhibit capacitation-associated increase in protein tyrosine phosphorylation implying that they may be involved in capacitation (present study and Carrera et al., 1996). This is likely since in many mammalian spermatozoa capacitation-associated increase in protein tyrosine phosphorylation is regulated by a cross talk between PKA, tyrosine kinase and tyrosine phosphatase. It is envisaged that AKAPs influence the above cross talk by partitioning at least a fraction of the PKA into a cytoskeletal compartment of the tail as in the case of mouse spermatozoa during capacitation (Visconti et al., 1997). This is the first report on a rodent AKAP that is tyrosine phosphorylated and also involved in capacitation. In fact, using anti-phosphoserine and anti-phosphothreonine antibodies, it was further confirmed that in hamster spermatozoa pro-AKAP83 and AKAP83 are phosphorylated only at the tyrosine residue.
Carrera et al (1996) observed that in human spermatozoa there was a dramatic increase in protein tyrosine phosphorylation after 1h of incubation in capacitation medium; this increase then persisted over time. Thus, it was hypothesized that these phosphorylated proteins may be important for some non-reversible event related to motility such as initiation of hyperactivation. A similar change was also observed in hamster spermatozoa except that in hamster it showed peak intensity of phosphorylation at 3 to 5h by which time hyperactivation and acrosome reaction were still on the rise. Therefore, in hamster it may be related to both hyperactivation and acrosome reaction. Harrison et al (2000) have demonstrated that in human spermatozoa AKAPs are involved in progesterone induced acrosome reaction and probably this is mediated through AKAP110 which has been localized both in the acrosomal region and tail of spermatozoa (Vijayaraghavan et al., 1999). In the present study, AKAP83 was localized specifically to the principal piece and was not detected in the head region and therefore, it may imply that phosphorylation of AKAP83 is related to hyperactivation rather than acrosome reaction. It is of interest to recapitulate that human AKAP82 is also localized only to the principal piece of the sperm tail (Turner et al., 1998). These observations taken together may also imply that different AKAPs may have different functions depending on their localization.

4.8 Role of Protein Serine/Threonine Phosphorylation in Hyperactivation, Capacitation and Acrosome reaction

Protein serine/threonine kinases such as protein kinase A (PKA) and protein kinase C (PKC) have been shown to regulate capacitation. However, little is known
about the changes in protein serine/threonine phosphorylation during capacitation that may play a crucial role in the regulation of this process. Furthermore, since it is established that there is a cross talk between PKA and protein tyrosine kinase/phosphatase pathway during capacitation, identifying the proteins which undergo differential phosphorylation at serine/threonine residues during capacitation will be very helpful in understanding the molecular basis of this process. So far, changes in protein serine/threonine phosphorylation has been studied only in human spermatozoa (Naz, 1999) wherein a set of 8-9 proteins in the range of mol. wt. from 15-200 kDa have been shown to undergo increased phosphorylation during capacitation at serine and/or threonine residues. One of these proteins was found to be the fertilization antigen-1 (FA-1) which was earlier shown to be tyrosine phosphorylated during capacitation. Furthermore, relocalization of these proteins during capacitation was demonstrated thus indicating that serine/threonine phosphorylation may have a role in sperm capacitation and/or acrosome reaction. Attempts were made in the present study to find out if serine/threonine phosphorylation is important for such maturational events in hamster.

The present study demonstrates a set of proteins that are phosphorylated at the serine/threonine residues in hamster caudal spermatozoa following in vitro capacitation. Many of these proteins (100, 66, 63 and 56 kDa) were phosphorylated at both the serine as well as the threonine residues whereas others (61, 53, 49, 35 and 32 kDa) were phosphorylated only at the threonine residues. Protein serine phosphorylation did not show any change with respect to the intensity of the bands during the incubation of the spermatozoa in a medium supporting capacitation until 7-
8 h except the 100 kDa protein which showed a time-dependent decrease. Most of the threonine phosphorylated proteins also showed the same level of phosphorylation till 7-8 h of incubation, except for two proteins (namely 63 kDa and 49 kDa proteins) which showed time-dependent increase during capacitation. These two proteins could be detected after 3 h and the intensity of their phosphorylation increased subsequently up to 7 h. It should be noted that protein tyrosine phosphorylation normally reaches saturation by 5 h. Further, unlike protein tyrosine phosphorylation, the threonine phosphorylation does not seem to correlate temporally with hyperactivation rather it appears to correlate with the acrosome reaction. It is possible that phosphorylation of these proteins are important for acrosome reaction and/or the downstream events. With this in view the effects of deprivation of media constituents namely BSA, PVA, \( \text{NaHCO}_3 \) and \( \text{Ca}^{2+} \) on serine/threonine phosphorylation was studied. It was observed that none of the media components had any effect on the serine/threonine phosphorylation. Thus it would appear that capacitation and protein serine/threonine phosphorylation are not related. It could also imply that the effectors effect capacitation by influencing components of a different signaling pathway and the enzymes regulating protein serine/threonine phosphorylation are independent of their effects. Therefore, a more directed strategy would be required to establish if any association exists between capacitation and protein serine/threonine phosphorylation. Therefore, specific inhibitors of the serine/threonine kinase and phosphatase were used to further investigate the role of serine/threonine phosphorylated proteins in capacitation.
When hamster spermatozoa were incubated in TALP containing H8 (N-(2-[methylamino] ethyl)-5-isoquinolinesulfonamide-hydrochloride), a serine/threonine kinase inhibitor (specific for PKA), it was observed that the percentage motility of spermatozoa was similar to the control up to 7 h, hyperactivation was delayed and acrosome reaction was inhibited. Earlier studies had also shown that H8 inhibited acrosome reaction in mouse (Furuya et al., 1992). Taken together it would appear that serine/threonine kinases are required for hyperactivation and acrosome reaction. This study also demonstrates that okadaic acid a serine/threonine phosphatase inhibitor (specific for protein-phosphatase-2A) also had no effect on the motility of hamster spermatozoa. But, the treated spermatozoa failed to undergo hyperactivation and acrosome reaction was temporally hastened. In human and fowl spermatozoa it was observed that calyculin, another protein phosphatase inhibitor and okadaic acid stimulated capacitation (Leclerc et al., 1996; Ashizawa et al., 1994). Though these studies have been done to find out if serine/threonine kinase and phosphatase (especially PP-1 and 2A) are involved in capacitation the difference in the exact event studied (i.e., motility, hyperactivation and capacitation) makes it difficult to make a conclusion about their precise involvement in capacitation of hamster spermatozoa. However, it is possible to conclude that serine/threonine kinase (particularly PKA) is a positive regulator of acrosome reaction whereas serine/threonine phosphatase (particularly PP-2A) is negative regulator of acrosome reaction. The importance of the delicate balance in protein serine/threonine with respect to capacitation is best demonstrated in hamster spermatozoa by Si and Okuno (1999) who showed that hyperactivation was inhibited by H8 but stimulated by calyculin.
With the existing evidences it is clear that serine/threonine phosphorylation is required for capacitation. Further, immunolocalization with anti-phosphoserine and anti-phosphothreonine antibodies showed that serine/threonine phosphorylated proteins are present in the head as well as in the tail thus implicating that these proteins may be involved in motility, hyperactivation, capacitation and acrosome reaction. Identification and characterization of these proteins which are differentially phosphorylated during capacitation would be very beneficial in establishing the exact role of the serine/threonine phosphorylated proteins in capacitation.

4.9 Working Model for Capacitation of Hamster Spermatozoa

Based on the information already available in the literature and the present findings it is clear that capacitation of hamster spermatozoa is regulated by a cross-talk between cAMP-dependent protein kinase A, protein tyrosine kinase(s) and protein tyrosine phosphatase(s). Media components such as BSA, Ca^{2+} and HCO_{3}^- modulate this cross-talk by influencing the enzymes that regulate cAMP levels required for PKA activity. Furthermore, certain phosphoproteins such as AKAP play a very crucial role in capacitation by bringing the kinase and/or phosphatase in close proximity to their substrates. Thus, it appears that protein phosphorylation/dephosphorylation is the key regulatory pathway for the process of capacitation. The model (Fig. 1) depicts the important components of signaling events and the possible targets of the modulators of capacitation of hamster spermatozoa.
Fig. 1 Working model demonstrating the signaling events hypothesized to be involved in capacitation of hamster spermatozoa (based on the present work and the reports from the other labs). Abbreviations: BSA, bovine serum albumin; PVA, polyvinyl alcohol; CHOL, cholesterol; AC$_{TM}$, transmembrane adenylyl cyclase; AC$_{S}$, soluble adenylyl cyclase; PDE, phosphodiesterase; PKC, protein kinase C; PKA, protein kinase A; AKAP, a kinase anchoring protein; P-AKAP, phosphorylated-AKAP; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase. (+) and (-) indicate the positive and negative regulation respectively.
5.0 Conclusions

1. Cauda epididymal spermatozoa of hamster exhibit capacitation-associated increase in protein tyrosine phosphorylation.

2. Capacitation-associated increase in protein tyrosine phosphorylation correlates positively with hyperactivation and acrosome reaction.

3. BSA alone and calcium and NaHCO₃ together can modulate capacitation of hamster spermatozoa by affecting protein tyrosine phosphorylation.

4. Hyperactivation of hamster spermatozoa appears to be linked to capacitation-associated increase in protein tyrosine phosphorylation.

5. Acrosome reaction of hamster spermatozoa appears to be independent of the capacitation-associated increase in protein tyrosine phosphorylation.

6. Capacitation-associated protein tyrosine phosphorylation in hamster spermatozoa is regulated both by PTK and PTP.

7. The major tyrosine phosphorylated protein in capacitated hamster spermatozoa is an AKAP.

8. Hamster AKAP is homologous to AKAP from other mammalian spermatozoa. It is synthesized as a precursor, has the PKA RII subunit binding domain and is localized to the principal piece of the tail.

9. Serine/threonine phosphorylated proteins may also be involved in capacitation, of hamster spermatozoa.