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Invited Review

Regulation of Calcium Transport in Mammalian Spermatozoa during Capacitation and Acrosome Reaction*

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Introduction

It is well documented that the acrosome reaction (AR) is a calcium-dependent event (Yanagimachi & Usui 1974, Singh et al. 1978, Fraser 1982, Meizel 1984, Yanagimachi 1988, Sidhu & Guraya 1989b). A net uptake of Ca^{2+} during capacitation and AR has been demonstrated (Singh et al. 1978, Peterson et al. 1983, Rufo et al. 1984, Vijayaraghavan & Hoskins 1988) and the experiments with the Ca^{2+} ionophore A23187 suggest that Ca^{2+} influx induces the occurrence of AR (Singh et al. 1978, Babcock et al. 1979; Aitken et al. 1984, Byrd & Wolf 1986, McLaughlin et al. 1989). Several studies demonstrated that Ca^{2+} also plays a significant role during fertilization (Metz & Monroy 1985, Guraya 1987, Schatten & Schatten 1989).

The regulation of Ca^{2+} transport in sperm is poorly understood. The kinetics of Ca^{2+} transport in spermatozoa change during their maturation in the epididymis; the ejaculated sperm are relatively impermeable to Ca^{2+} probably because of the presence of caltrin in seminal plasma that inhibits Ca^{2+} accumulation in spermatozoa (Babcock et al. 1979, Rufo et al. 1984, Lewis et al. 1985). The permeability to Ca^{2+} increases during sperm passage in the female reproductive tract (Meizel 1985). Various components believed to be involved in the process of Ca^{2+} transport such as Ca^{2+}-ATPase, Na^+, K^+-ATPase, Na^+/Ca^{2+} antiporter, ion channels, Ca^{2+}-binding proteins, calmodulin and calmodulin-like protein have been demonstrated in intact mammalian spermatozoa and also in their isolated plsma membrane vesicles (Gordon 1973, Forrester & Bradley 1980, Jones et al. 1980, Vijayasarathy et al. 1980, Feinberg et al. 1981, Ashraf et al. 1982, Peterson et al. 1983, Moore & Dedman 1984, Mrsny et al. 1984, Weinman et al. 1986, Aitken et al. 1988, Vijayaraghavan & Hoskins 1988, Sidhu & Guraya 1989b, Cox & Peterson 1989, Leclerc et al. 1990). However, very divergent views prevail about their localization in spermatozoa and also in their mode of action particularly their modulations during sperm capacitation and AR.

The major aim of the present review is to integrate the available information on all aspects believed to be important for regulating Ca^{2+} transport in spermatozoa particularly during capacitation and AR. Attempts are also made to propose a unified hypothesis explaining Ca^{2+} regulation in spermatozoon during capacitation and AR.

Results and Discussion

Role of Calcium in Capacitation and AR

Capacitation is considered to involve a series of incompletely understood subcellular, biochemi-

*Dedicated to my teacher Prof. S.S. Guraya for his untiring and sincere contributions to science

cal and molecular changes that prepare the spermatozoa for AR (Meizel 1984, 1985, Metz & Monroy 1985, Guraya 1987, Sidhu 1988, Sidhu & Guraya 1989a). Most of the current studies have attempted to elucidate various biochemical or molecular changes occurring during capacitation that prepare the spermatozoa for AR. However, the present state of knowledge still does not allow us to distinguish clearly the events during capacitation and AR, but the former is considered to be the prerequisite for the latter. No unified view of the biochemical or molecular mechanism of capacitation has yet emerged from the results of numerous *in vitro* studies (Sidhu and Guraya 1989a). Most of the observations point to the conclusion that capacitation involves two important changes at the molecular level in spermatozoa: (i) sperm surface alternation and/or intramembranal molecular mobility to facilitate Ca^{2+} influx, and (ii) changes in sperm energetics via alterations in oxygen uptake and glucose utilization manifested in the form of changes in pattern of flagellar beat (hyperactivated motility). These molecular changes during capacitation ensure the timely occurrence of AR. The release of hydrolytic enzymes from the acrosome during AR and the generation of greater thrust in sperm showing hyperactivity during capacitation greatly facilitate the entry of spermatozoa through egg investments for fertilization.

It is generally considered that all the biochemical and molecular changes on the surface of spermatozoa and intramembranal molecular alterations during capacitation facilitate the influx of Ca^{2+}. The molecular model of sperm capacitation put forth by O'Rand (1979) shows that the plasma membrane prior to capacitation (figure 1A) has four classes of molecules involved in the sperm surface change: (i) glycoproteins that are mobile within the

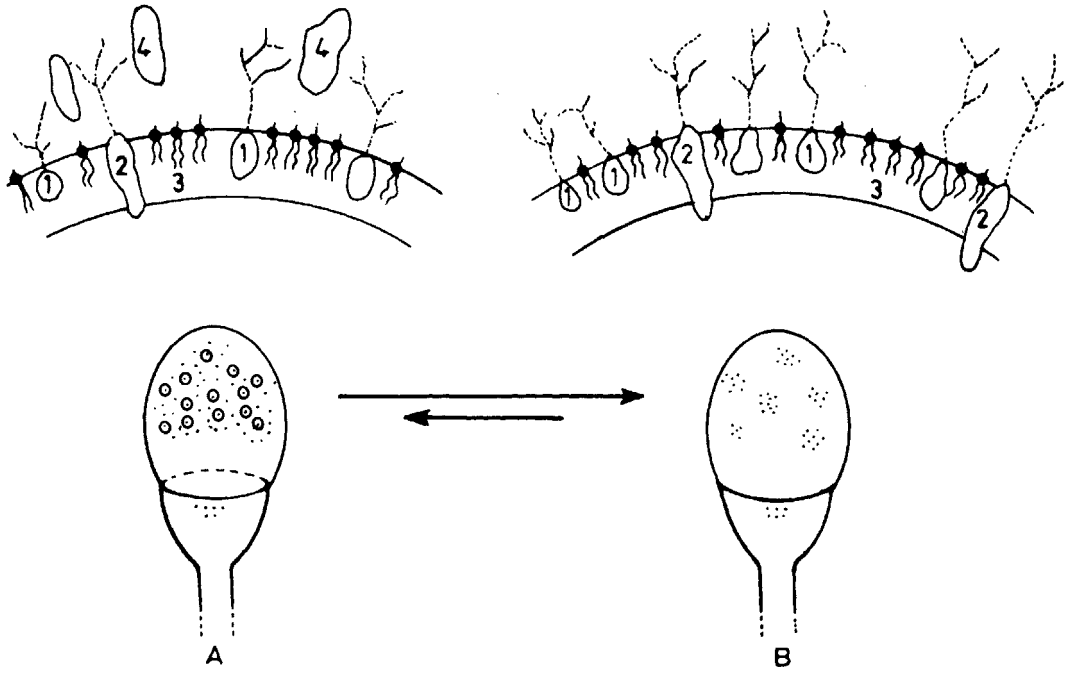

Figure 1 A model for the surface changes in sperm membranes associated with capacitation. (A) Prior to capacitation; (B) after capacitation. Four classes of molecules are shown: 1, mobile glycoproteins; 2, nonmobile glycoproteins; 3, glycolipids; 4, peripheral components. The lower sketches show the surface pattern expected for intrinsic mobile glycoproteins (class 1) in association with peripheral components (class 4) before capacitation (A) and without peripheral components after capacitation (B). From O'Rand (1979)

plane of the membrane, (ii) nonmobile glycoproteins, (iii) glycolipids, and (iv) peripheral membrane components. After capacitation the relationship among the four classes of molecules changes (figure 1B). Peripheral components are modified or removed, and mobile and nonmobile classes (i and ii) are reassociated. Thus, a protein poor, high-fluidity area ready for membrane fusion may coexist with protein-rich areas of decreased fluidity. This is consistent with the pattern of membrane fusion seen during AR. Calcium has also been shown to modulate sperm metabolism, respiration and motility during capacitation. A relationship exists between Ca^{2+}, c-AMP and calmodulin during capacitation in mammalian spermatozoa (Morton & Albagli 1973, Garbers & Kopf 1980, Rufo et al. 1984, Sidhu et al. 1984, Nagae & Srivastava 1986, Sidhu & Guraya 1989a, b) that facilitates the onset of AR.

There is general concensus that Ca^{2+} is essential for the occurrence of AR in spermatozoa. The development of a synchronous system for the induction of AR in guinea pig by Yanagimachi and Usui (1974), which involves incubating sperm in Ca^{2+} free medium for 10 hr and then adding Ca^{2+} when 40-80% of sperm show AR, gives support to the role of Ca^{2+} in AR. Similarly, several studies utilizing calcium ionophore A_{23187} have demonstrated the role of Ca^{2+} in AR (Singh et al. 1978, Babcock et al. 1979, Aitken et al. 1984, Byrd & Wolf 1986, McLaughlin et al. 1989). Singh et al. (1980) suggested that although uptake of Ca^{2+} by sperm is required for the AR, a high concentration of Ca^{2+} exerts an adverse effect on the survival of spermatozoa that have undergone AR. They proposed that some additional mechanism may operate to protect the sperm by limiting the entrance of Ca^{2+} during capacitation *in vivo*. The likely candidate for such a control would be the Ca^{2+}-ATPase. It has been shown that the uptake of $^{45}Ca^{2+}$ occurs slightly before AR and the Ca^{2+} uptake is a voltage-dependent and *p*H-sensitive mechanism (Babcock & Pfeiffer 1987). Sr^{2+} but not Mg^{2+} or Zn^{2+} can replace Ca^{2+} in inducing AR (Yanagimachi & Usui 1974, Meizel & Lui 1976, Mortimer 1986, Fraser 1987, Mortimer et al. 1986, 1988). The significance of Ca^{2+} in inducing AR is not very clear. Sidhu & Guraya (1989a) proposed a model, explaining the role of Ca^{2+} in AR (see figure 2).

Components of Sperm Involved in Calcium Transport

ATPases/calcium pump: Ca^{2+}-ATPase and Na^{2+}, K^{+}-ATPase are involved in regulating Ca^{2+} homoeostasis in various cells (Carafoli 1987, Kaczorowski et al. 1989) and these ATPases are reported to be present in mammalian spermatozoa (Meizel 1984, Sidhu & Guraya 1989a, Roldan & Fleming 1989). Ca^{2+}-dependent ATPase is reported to be present on the outer acrosomal membrane of mammalian spermatozoa (Gordon et al. 1978) that pumps Ca^{2+} into the acrosome, thus stimulating AR. However, inward-directed Ca^{2+} pump is present only in sarcoplasmic reticulum and not in any other somatic cells (Stekhoven & Bonting 1981). Other workers demonstrated the presence of an outward-directed Ca^{2+} pump Ca^{2+}-ATPase in the head and flagellar membranes of bull, buffalo bull, ram and boar spermatozoa (Vijayasarathy et al. 1980, Ashraf et al. 1982, Breitbart et al. 1983, 1984, Peterson et al. 1983, Sidhu & Guraya 1989b). Bull sperm Ca^{2+}-ATPase is Mg^{2+}-independent (Vijayasarathy et al. 1980). However, other reported Ca^{2+}, Mg^{2+}-ATPase regulating Ca^{2+} in bull and buffalo bull spermatozoa (Breitbart et al. 1984, Sidhu & Guraya 1989b). These studies suggest that inhibition of Ca^{2+}-ATPase should facilitate Ca^{2+} influx. Santos-Sacchi and Gordon (1982) showed that depletion of ATP inhibits Ca^{2+}-ATPase in guinea pig spermatozoa, which facilitates the Ca^{2+} influx required for AR. Rufo et al. (1984) reported caltrin, an inhibitor of sperm Ca^{2+} transport in bull seminal plasma. Regulation of sperm Ca^{2+}-ATPase by calmodulin and calmodulin-like protein has also been reported (Peterson et al. 1983, Vijayaraghavan & Hoskins 1988, Aitken et al. 1988, Sidhu & Guraya 1989a, b).

Ouabain-sensitive Na^{+}, K^{+}-ATPase is reported in rat, rabbit, hamster, bull and boar spermatozoa (Gordon 1973, Ashraf et al. 1982, Mrsny & Meizel 1982, Mrsny et al. 1984, Breitbart et al. 1984). Inhibition of capacitation and AR of hams-

ter spermatozoa by Ouabain indicates the role of Na^+, K^+-ATPase in these events (Meizel 1984). A relationship exists between Na^+, K^+-ATPase activity, c-GMP and AR in hamster spermatozoa (Mrsny et al. 1984). It is proposed that K^+ influx is important during capacitation and AR in hamster spermatozoa (Mrsny & Meizel 1982), in rat spermatozoa, however, higher levels of K^+ decrease AR (Rogers et al. 1981, Fraser 1983). How Na^+ K^+-ATPase is involved in AR is not clear. K^+ influx may be coupled to H^+ efflux, resulting in intraacrosomal increase in *p*H, the latter is important for AR. Influx or efflux of either Na^+ or K^+ may also change the membrane potential and thus facilitate AR. Mrsny and Meizel (1985) showed that taurine and hypotaurine, sulfur-containing β-amino acids inhibit Na^+, K^+-ATPase in hamster spermatozoa, and thus stimulates Na^+ accumulation in spermatozoa that can be exchanged with extracellular Ca^{2+} as Ca^{2+}/Na^+ antiport has been demonstrated in sperm (Bradley & Forrester 1980, Rufo et al. 1984) and the Ca^{2+} is necessary for sperm motility and AR (Nelson 1985, Sidhu 1988).

Calcium channels: The presence of ion-selective channels in the plasma membrane of invertebrate spermatozoa is firmly established (Kazazaglou et al. 1985, Lee & Garbers 1986, Guerrero et al. 1987, Toowicharanont & Shapiro 1988). Similar studies about such channels have been initiated recently in mammalian spermatozoa (Babcock 1988, Florman & Babcock 1988, Vijayaraghavan & Hoskins 1989, Fraser & McIntyre 1989, Cox & Peterson 1989, Juneja et al. 1990a, b). These studies measured internal Ca^{2+} and H^+ concentrations using fluorescence probes during capacitation, however, Cox & Peterson (1989) for the first time used patch clamp techniques in boar spermatozoa which clearly demonstrated the presence of channels in the plasma membrane of boar spermatozoa capable of conducting Ca^{2+} ions. Patch clamp techniques have been used extensively in sea urchin spermatozoa for detecting ion channels (Guerrero et al. 1987, Toowicharanont & Shapiro 1988). Studies using channel blocker such as La^{3+}, nitrendipine and verapamil demonstrated that several types of channels very likely exist in boar sperm membrane. One type resembles L-channels found in abundance in skeletal muscle (sensitive to nitrendipine) (Cox & Peterson 1989). Other may represent a large calcium conductance describe in sea urchin spermatozoa. This reflects the regional heterogeneity of the sperm surface and the different functions of its several domains (Peterson & Russell 1985). In boar sperm plasma membrane channels, the frequency of closing increases by verapamil, but this effect was short-lived (Cox & Peterson 1989). In guinea pig sperm plasma membrane vesicles, verapamil, however, has been shown to stimulate Ca^{2+}-uptake and Ca^{2+}-ATPase (Juneja et al. 1990a, b). Recently it has been shown that mammalian sperm plasma membrane contains a calcium/phosphate symporter, a phosphate-independent calcium carrier and a calcium-independent phosphate carrier, calcium uptake into ejaculated ram spermatozoa is enhanced by addition of extracellular phosphate. Under identical conditions extracellular calcium stimulates uptake of phosphate by cells. Both phosphate and calcium uptake are comparably inhibited by sulphahydryl reagent, mersalyl. The calcium channel blocker, verapamil inhibits Ca^{2+} uptake in the presence or absence of extracellular phosphate. The phosphate-dependent calcium transport mechanism is more sensitive to verapamil than in the phosphate-independent transporter (Zarca et al. 1988).

Our knowledge about ion channels in sea urchin sperm is very exhaustive (Guerrero et al. 1987) and the receptors for phenylalkylamine and nitrendipine have been detected in sperm membranes. Studies using channel antagonists have demonstrated the role of these channels in sperm acrosome reaction (Schackmann et al. 1978, Kazagzaglou et al. 1985). Our knowledge about ion channels in mammalian spermatozoa is very scanty. Recently Lee & Storey (1988), however, showed that there is rapid influx of Ca^{2+} during the last phase of AR in mouse spermatozoa. Similarly based upon the use of various ion channel blockers and intracellular levels of Ca^{2+}, it has been suggested that selective increase in Ca^{2+} uptake during capacitation in bull sperm involves activation of voltage-dependent Ca^{2+} channel possible under the control of a G protein

(Babcock & Pfeiffer 1987, Babcock 1988, Florman & Babcock 1988). It is also shown that solubilized zonae induce the AR in bull sperm (Florman & Babcock 1988) and pertussis toxin blocks the rises of intracellular Ca^{2+} and *p*H that are part of this signal pathway.

Similarly, Fraser and McIntyre (1989) suggested that in mouse sperm, Ca^{2+} channels similar to those termed voltage-sensitive in other cell types may exist and play an important role in Ca^{2+} transport during late stage of capacitation. Thomas & Meizel (1989), however, observed no affect of verapamil on the follicular fluid-induced AR in human spermatozoa. In sea urchin spermatozoa, fucose sulphate glycocojugate from egg jelly stimulates Ca^{2+} influx probably via a voltage-operated Ca^{2+} channel (VOCC) (Schackmann et al. 1978, 1981). In mammalian spermatozoa, the effects of VOCC antagonists on AR are contradictory (Roldan et al. 1986). Apart from VOCCs, the regulation of Ca^{2+} influx through the plasma membrane is poorly understood. A variety of ligands, however, can control Ca^{2+} influx by means other than VOCCs (Meldolesi & Pozzan 1987). These include (i) second-messenger-operated Ca^{2+} channel (SMOCCs), in which receptor and ion channels are separate entities coupled by a diffusible second messenger, and (ii) receptor-operated Ca^{2+} channels (ROCCs) in which the receptor and ion channel coexist as an individual functional unit. Nothing is known about the existence and operation of these kinds of channels in mammalian spermatozoa and need to be explored in future studies.

Sodium-calcium antiporter : One of the most important processes involved in regulating Ca^{2+} homeostasis in both excitable and non-excitable tissue is the Na-Ca exchange (Na-Ca antiporter) mechanism. Since Na-Ca exchange is a completely reversible, electrogenic transport reaction, operation of the carrier is controlled by the magnitude and polarity of transmembrane electric potentials and ionic gradients. Therefore, Na-Ca exchange could provide either net cellular efflux or influx of Ca^{2+}, depending on prevailing physiological conditions. Thus, Na-Ca exchange reaction is electrogenic, bidirectional and function with a stoichiometry of 3 Na^{+}:Ca^{2+} and is kinetically symmetrical (Kaczorowski et al. 1989). The possibility of the existence of similar Na-Ca exchange mechansim in mammalian spermatozoa could be envisaged based upon the available information about the rapid influx of Ca^{2+} observed during AR (Yanagimachi 1981, 1988), involvement of Na^{+}, K^{+}-ATPase in sperm capacitation (Mrsny et al. 1984, Mrsny & Meizel 1985) and decrease in membrane potential during capacitation (Calzada et al. 1988). Only a few studies using very controlled conditions have demonstrated the presence of Na-Ca exchange mechanism in the membrane vesicles from hamster, boar, ram and bull spermatozoa (Bradley & Forrester 1980, Ashraf et al. 1982, Rufo et al. 1984, Ruknudin 1989, Sidhu, unpublished observation). Caltrin, a Ca^{2+} transport inhibiting factor from seminal plasma (Rufo et al. 1984) is considered to interfere with Na-Ca exchange mechanism in bovine spermatozon (Rufo et al. 1984). However, a certain degree of caution needs to be exercised in interpretation of data for a Na-Ca exchange mechanism in sperm because (i) such studies have been carried out in isolated membrane vesicles which may be of mixed polarity i.e., they may have both right-side out and inside-out vesicles and they may have lost regulatory features found in the intact cell, (ii) no known specific inhibitor for Na-Ca exchange mecanism has been used in these studies. But nevertheless, these and the future studies on Na-Ca exchange mechanism in sperm would be of paramount-importance because this mechanism is bidirectional, and changes directions (Ca influx or efflux) with change in membrane potential and the latter has been demonstrated in human spermatozoa (Calzada et al. 1989).

Inositol triphosphate (IP_3)/Diacylglycerol (DG): Sperm acrosome reaction is often compared and found similar to exocytosis in other systems (Sidhu & Guraya 1989a). In exocytosis a complex interplay exists between Ca^{2+}, lipids and protein components (Nishizuka 1984, De Lisle & Williams 1986). The breakdown of membrane polyphosphoinositides to inositol triphosphate and diacylglycerol as second messengers, triggering the process of exocytosis via Ca^{2+} is frequently reported (Berridge

1987). However, as yet little advantage is taken of these new discoveries in studies of the molecular events of the mammalian sperm AR. Recently, some attempts have been made to interrelate the inositol phosphates, diacylglycerol levels to Ca^{2+} influx during AR in sea urchin and mammalian spermatozoa (Lee et al. 1987, Domino & Garbers 1988, Roldan & Harrison 1989, Thomas & Meizel 1989, Visconti & Tezon 1989). Among other Ca^{2+}--dependent reactions, the breakdown of phospholipids by activation of membrane phospholipase C and A_2 occurs during the capacitation process of spermatozoa (Lui & Meizel 1979, Dravland & Meizel 1982). The products of these reactions are proposed to play a role in the uptake or mobilization of Ca^{2+} required for AR (Fleming & Yanagimachi 1981, Meizel 1984). Recently the presence of large quantity of phospholipase (specific for phosphoinositides) and the release of diacylglycerol during A_{23187}-induced AR is shown in human spermatozoa (Ribbes et al. 1987, Benet et al. 1987). Similarly, loss of phosphatidyl inositol and the polyphosphoinositides is observed in spermatozoa undergoing AR (Nikolopoulo et al. 1986, Benet et al. 1987, Roldan & Harrison 1989). But the turnover of the phosphoinositides in direct response to physiological agonist is not yet demonstrated in mammalian spermatozoa. Recently Thomas & Meizel (1989) demonstrated that phosphoinositide hydrolysis occurs in human sperm when stimulated by a potentially physiological stimulus i.e. follicular fluid or progesterone. They also proposed that progesterone binds to a cell-surface receptor which in turn activates phosphoinositide breakown leading to inositide triphosphate-mediated Ca^{2+} mobilization. In mammalian spermatozoa the breakdown of polyphosphoinositides to inositol phosphates (inositol triphosphate) and diacylglycerol is demonstrated (Roldan & Harrison 1989, Thomas & Meizel 1989). One can thus speculate their possible role in AR. In other cell systems, the role of inositol triphosphate is to mobilize Ca^{2+} from internal reserves such as in endoplasmic reticulum (Berridge 1987), although recently it has been proposed that inositol 1, 3, 4, 5-tetrakisphosphate together with inositol triphosphate may promote Ca^{2+} entry into the cell via plasma membrane associated pathways (Irvine & Moor 1987). Such phosphoinositide breakdown usually occurs through receptor-mediated phospholipase C activation as is also shown in human spermatozoa by Thomas & Meizel (1989). Diacylglycerol, the other biproduct of phosphoinositol breakdown is also ascribed a second-messenger role in other cell systems via its stimulation of protein kinase C (Nishizuka 1984). However, the presence of protein kinase C in mammalian spermatozoa is not conclusive (Roldan & Harrison 1989). In other cells, this enzyme is activated by either by 1, 2-diacylglycerol generated by ligand-induced, receptor mediated polyphosphoinositide hydrolysis or by a variety of exogenous agents such as phorbol esters (Brock et al. 1985, Sharkey & Blumberg 1986, Berridge 1987). Lee et al. (1987) showed that phorbol esters stimulate zona-induced AR in mouse spermatozoa, suggesting an intermediate role of protein kinase C in AR. Similarly Visconti & Tezon (1989) suggested the involvements of protein kinase C activity in the regulation of cAMP levels in hamster spermatozoa during capacitation. This stimulation is dependent on intracellular Ca^{2+} and probably is not linked to the process of the AR. Roldan & Harrison (1989) also denied a protein kinase C-stimulating role for diacylglycerol at least downstream of Ca^{2+} entry in the AR of ram, boar, guinea pig and human spermatozoa. But diacylglycerol and its product phosphatidic acid are shown to be fusogenic (Sundler & Papahadopoulos 1981, Das & Rand 1984). On the other hand, diacylglycerol is shown to increase the susceptibility of phospholipids to attack by phospholipases (Dawson et al. 1984); the fusability of membrane could be enhanced via production of lysophosphatides by either phospholipase A_2 (Sidhu & Guraya 1989a) or by modification of sperm lipid configuration or content (Fleming et al. 1982, Sidhu & Guraya 1985, 1990, Jones & Plymate 1989).

Proteins Involved in Regulating Calcium Transport in Spermatozoa

Calmodulin and calmodulin-binding proteins: Calmodulin is an ubiquitous Ca^{2+} binding pro-

tein which regulates a large number of enzymes (Cheung 1980). Immunofluorescence techniques have shown the presence of calmodulin in the head region of mammalian spermatozoa (Jones et al. 1980), and closely localized to the region containing phospholipase A_2 (Weiman et al. 1986). The latter is modulated by calmodulin (Moskowitz et al. 1983). Recently Langlais et al. (1988) demonstrated that sperm phospholipase A_2 is actually a calmodulin binding protein in bull spermatozoa. Several calmodulin-binding and calmodulin-like protein has been identified in bovine (Olson et al. 1985, Leclerc et al. 1990), buffalo (Sidhu & Guraya 1989b), hamster (Moore & Dedman 1984), boar (Peterson et al. 1989) and human spermatozoa (Aitken et al. 1988). It, therefore, seems possible that calmodulin is involved directly or through its Ca^{2+}-binding properties in the capacitation/AR processes. Calmodulin antagonists have been shown to induce AR in guinea pig and boar spermatozoa (Peterson et al. 1983, Berruti et al. 1986, Nagae & Srivastava 1986). Peterson et al. (1989) identified six calcium-binding and calmodulin-binding proteins from plasma membrane of boar spermatozoa which showed some modulations during *in vitro* capacitation indicating their role in regulating events involving calcium in mature spermatozoa. They also isolated a single high molecular weight (300 kd) calcium binding protein from boar seminal plasma that binds specifically to the plasma membrane overlying the principal segment and is removed from sperm during capacitation. Sidhu & Guraya (1989b) isolated a calmodulin-like protein (CLP) from buffalo seminal plasma that stimulates Ca^{2+}, Mg^{2+}-ATPase of intact buffalo sperm as well as the enzyme in purified plasma membrane preparations from spermatozoa. The enzyme stimulation was counteracted by anticalmodulin drugs. Indirect immunofluorescent study using antibodies against CLP showed the localization of CLP on the surface of spermatozoa which was removed during capacitation *in vitro* (Sidhu, unpublished observations). They proposed that CLP regulates Ca^{2+} levels during capacitation and AR in spermatozoa. Leclerc et al. (1989) using ^{125}I-labelled calmodulin gel overlay procedure detected the presence of 14 calmodulin-binding proteins in bull spermatozoa. One of these binding proteins (170 kd) required Ca^{2+} for binding to calmodulin while another (17 kd) bound only in the absence of Ca^{2+}. Other binding proteins, e.g. that of 62 kd, exhibited greater binding when Ca^{2+} was present for the overlay, while other, such as those between 14 and 30 kd exhibited greater binding to calmodulin in the presence of 1 m MEGTA. Using heparin to induce capacitation in bull spermatozoan, they have shown a decrease in the binding of calmodulin to three sperm calmodulin binding proteins of 28, 30 and 49 kd (Leclerc et al. 1989, 1990). Since heparin induces Ca^{2+} uptake in sperm cells (Handrow et al. 1986) and the studies of Leclerc et al. (1989, 1990) showed a lesser expression of calmodulin binding to the 28, 30 and 49 kd calmodulin binding proteins with increasing concentrations of heparin. It appears conceivable that these two phenomena are related. This is supported by the observation that these 3 calmodulin binding proteins exhibited greater binding to calmodulin when the overlay procedure was performed in the absence of Ca^{2+}. Thus, the decreae in the binding of calmodulin to these calmodulin binding proteins might result from the capacitation process itself (Leclerc et al. 1990). However, the mechanism of decreased binding of calmodulin to calmodulin binding proteins is not clear as yet. Changes in calmodulin binding can be effected through modifications of binding proteins probably by phosphorylation as Olson et al. (1985) showed that proteins of the outer acrosomal membrane in the 12-34 kd range can be phosphorylated in a cAMP- and Ca^{2+} independent manner. In human spermatozoa Aitken et al. (1988) reported the presence of four calmodulin binding proteins of which two bound calmodulin in a calcium independent and two other in a calcium-dependent manners with molecular weight of 59.5 kd, 44.8 kd and 32 kd, 22 kd respectively in the purified membrane fractions. Using anticalmodulin drugs i.e., trifluoperazine, they showed a marked increase in the concentration of intracellular calcium as also shown in board spermatozoa by Peterson et al. (1983). These results indicate the presence of a calmodulin-dependent Ca^{2+}-ATP-

ase in the sperm plasma membrane which is responsible for maintaining calcium homeostasis as also demonstrated in human and buffalo spermatozoa (Bradley & Forrester 1980, Sidhu & Guraya, 1989b). Aitken et al. (1988), however, failed to demonstrate the presence of any major calcium-dependent, calmodulin binding or the binding of extracellular calmodulin to the sperm surface. Similarly Peterson et al. (1983) showed that in boar sperm membranes, the ATP-dependent pumps are not controlled by calmodulin. In light of these findings, the most plausible explanation for the rise in Ca^{2+} observed with high doses of antagonists by Aitken et al. (1988) in human spermatozoa may be a nonspecific interference with the plasma membrane Ca^{2+}-pump ATPase, as described for the purified erythrocyte Ca^{2+}-ATPase (Vincenzi et al. 1982). But in buffalo spermatozoa, the binding of CLP has been demonstrated using indirect immunofluorescence (Sidhu, unpublshed observations). In bull spermatozoa, trifluoperazine, a calmodulin antagonist, inhibits the AR induced by proteoglycan (Lenz et al. 1982). Similarly Aitken et al. (1988) showed that lower concentrations of antagonists decreased the sperm/oocyte fusion as well as the penetration rate of zona-free hamster eggs suggesting the role of calmodulin and calmodulin binding proteins in sperm/oocyte fusion. Recently Leclerc et al. (1990) also showed that in bull spermatozoa during capacitation, the binding of calmodulin to calmodulin binding proteins decreases thus rendering calmodulin available for either the AR or the fusion or penetration of the oocyte. However, the mechanism by which sperm capacitation reduces the binding of calmodulin to calmodulin binding proteins is yet not revealed, probably sperm proteases or protein phosphorylation might modify the calmodulin binding protein during capacitation.

Caltrin/seminal plasmin: It is a well established fact that there is a rapid influx of Ca^{2+} in spermatozoa immediately before the onset of AR (Yanagimachi 1981, 1988) and the Ca^{2+} is very important for AR (Sidhu & Guraya 1989a). The regulation of Ca^{2+} transport in sperm is, however, poorly understood. It is also demonstrated that mammalian epididymal spermatozoa but not ejaculated spermatozoa can accumulate Ca^{2+} *in vitro* (Singh et al. 1978, Babcock et al. 1979, Tamblyn et al. 1979, Peterson et al. 1979). The inability of ejaculated bovine spermatozoa to accumulate Ca^{2+} despite of the fact that seminal fluid that surround these spermatozoa contain abundant calcium, led to the concept that a component of bovine seminal fluid presumably interacts with the surface membrane of ejaculated sperm to prevent or delay accumulation of Ca^{2+} by these cells (Babcock et al. 1979). The seminal calcium transport inhibitor is shown to be a single protein consisting of a single polypeptide chain of approxiamtely 108 amino acid residues having an isoelectric point of 8.3 as it is rich in basic amino acid residues. The protein contains no carbohydrate and has an apparent molecular weight of 10,000. These properties serve to distinguish seminal calcium transport inhibitor from other previously characterized seminal plasma proteins namely, forward motility protein, seminal plasmin, calcium binding protein, and calmodulin-like protein (Lukac et al. 1976, Acott & Hoskins 1978, Reddy & Bhargava 1979, Forrester & Bradley 1980, Sidhu & Guraya 1989b). Lewis et al. (1985) designated this seminal protein as 'caltrin'. The amino acid sequence analysis of caltrin showed its homology with seminal plasmin, a protein possessing antibacterial activity (Reddy & Bhargava 1979, Scheit et al. 1979, Theil & Scheit 1983, Sivaji et al. 1990). Seminal plasmin has properties characteristics of caltrin, that is the ability to inhibit calcium transport into bovine epididymal spermatozoa (Lewis et al. 1985).

San Augustin et al. (1987) showed that epididymal spermatozoa unlike ejaculated spermatozoa accumulate Ca^{2+} and contain no caltrin as is demonstrated by their inability to bind anticaltrin IgG, the latter is localized over the acrosome and principal tail region, but not to the midpiece of bovine spermatozoa. Recently, Breitbart et al. (1990) demonstrated that caltrin inhibits Ca^{2+} accumulation by intact bovine spermatozoa, whose plasma membrane has been permeabilized with filipin, and isolated rat liver and beef heart mitochondria. The sperm plasma membrane, otherwise, is imper-

meable to caltrin as it does not alter mitochondrial respiration in intact spermatozoa. Caltrin does not have appreciable affinity for Ca^{2+}, and thus the mechanism of its action does not involve chelation of extracellular Ca^{2+}. It is considered that caltrin specifically inhibits bovine sperm plasma membrane Ca^{2+} flux probably at Na^{+}/Ca^{2+} antiporter site (Rufo et al. 1984, Breitbart et al. 1990). Thus it can be concluded that caltrin is added to the surface membrane of sperm at the time of ejaculation which results in delayed uptake of calcium into these cells. After insemination, the inhibitor protein is presumably removed or modified in response to unknown component present in the female reproductive tract. In this way sperm are prevented from accumulating Ca^{2+} until such time as sperm and eggs are in close contact (Rufo et al. 1984). Vijayaraghavan and Hoskins (1988) demonstrated that calcium-inhibiting effect of caltrin in seminal plasma has different kinetics in caput and cauda sperm unlike in cauda sperm, the kinetics of calcium uptake in the presence of bovine seminal plasma (source of caltrin) into caput sperm showed a biphasic response, a stimulation of uptake at 1 to 15 min and inhibition of uptake after this time. They also demonstrated that preincubation of caput sperm with caudal epididymal fluid eliminated the biphasic calcium uptake effect induced in caput sperm by bovine seminal plasma. They concluded that bovine seminal plasma contains a low molecular weight factor derived from caudal epididymal fluid that interacts with developing sperm before the binding of caltrin to sperm can prevent further calcium uptake.

Mechanisms of Calcium Transport in Mammalian Spermatozoa

Various studies have clearly revealed that Ca^{2+} transport in sperm plays a significant role during AR (Yanagimachi 1982, 1988, Sidhu & Guraya 1989a) and in sperm motility (Nelson 1985). The kinetics of Ca^{2+} transport in sperm changes during their maturation in the epididymis; the ejaculated sperm are relatively impermeable to calcium but permeability to Ca^{2+} increases during their passage in the female reproductive tract. The regulation of Ca^{2+} transport in sperm still continues to be poorly understood phenomenon and thus no unified view of the mechanism of Ca^{2+} transport in spermatozoa can be proposed. However, various investigators working to elucidate the mechanism of Ca^{2+} transport in sperm have their working hypotheses as discussed here.

The first hypothesis for sperm Ca^{2+} transport mechanism was proposed by Gordon group (Gordon 1973, Gordon et al. 1978) based largely on their observations of ATPase activity in guinea pig, rabbit and human spermatozoa using cytochemical techniques at the electron microscopic level. A Ca^{2+}-independent, sensitive to ouabain is present on the sperm plasma membrane. This ATPase is inactivated by a decapacitation factor (DF) from seminal plasma and is activated when DF is removed. The activated ATPase transports extracellular Ca^{2+} to the space between the plasma and outer acrosomal membranes. This intracellular Ca^{2+} activates another Ca^{2+}-dependent ATPase on the outer acrosomal membrane, which then transports Ca^{2+} into the acrosome. This intraacrosomal Ca^{2+} starts the cascade of events (see subsequent discussions) leading to AR. This hypothesis has its limitations (i) the Ca^{2+} independent ATPase reported on the plasma membrane of spermatozoa was shown to be inhibited by ouabain (Gordon 1973) strongly suggesting that it was a Na^{+}, K^{+}-ATPase rather than a Ca^{2+} transport enzyme and (ii) there is as yet no biochemical evidence for an inward directed Ca^{2+}-ATPase pump on the outer acrosomal membrane; instead acrosomal membrane Ca^{2+}-ATPase in other mammalian species have been shown to be outward direct Ca^{2+} pump (Meizel 1984). Similarly, Meizel (1978) proposed in hamster spermatozoa that Ca^{2+} flows into the space between the plasma and outer acrosomal membranes probably mediated by catecholamine, ATP, ATPase adenylate cyclase, cAMP etc. (Meizel 1984). This leads to increased Ca^{2+} uptake by the acrosome due to stimulation of a Ca^{2+}-dependent ATPase as suggested by Gordon (1973). This hypothesis has the same limitations as suggested for Gordon hypothesis for Ca^{2+} transport in spermatozoa.

The hypothesis of Green (1978) in guinea pig spermatozoa assumes the presence of a receptor in the plasma membrane which is normally occupied by an antagonist. This antagonist is removed during capacitation and the stimulus ligands (from follicular fluid or zona pellucida) occupy the receptor. The triggered-receptor allows an influx of extracellular Ca^{2+}. The receptor-operted components of sperm plasma membrane responsible for conducting Ca^{2+} transport are yet to be identified in mammalian spermatozoa although calmodulin operated Ca^{2+}-ATPase and caltrin-regulated Na^{+}/Ca^{2+} antiporters have been reported. Similarly hypothesis has also been proposed by Yanagimachi (1981), who assumes that adsorption of inhibitory substances from seminal plasma occurs on the plasma membrane intercalated protein particles responsible for conducting Ca^{2+} and this inhibition is removed during capacitation in the female reproductive tract by decoating of inhibitory substances from sperm surface thus facilitating Ca^{2+} entry for the occurrence of AR. The accumulated intracellular Ca^{2+} inhibits the Mg^{2+} dependent ATPase reported on the plasma and the outer surface of the outer acrosomal membrane of guinea pig spermatozoa (Usui & Yanagimachi 1986). Mg^{2+} ATPase may function as a proton-translocating enzyme in the spermatozoa with intact acrosome to maintain an acidic intracellular *p*H (Working & Meizel 1982), when the enzyme is inactivted by Ca^{2+}, resulting H^{+} efflux, causing intracellular *p*H to rise that may facilitate the occurrence of AR (Meizel 1984).

Lardy group working on bovine spermatozoa demonstrated the presence of a low molecular weight basic protein called caltrin from seminal plasma that binds the sperm plasma membrane and inhibits the accumulation of Ca^{2+} in the cell probably by blocking Na^{+}/Ca^{2+} antiporter and this inhibition is removed during capacitation, probably by dislodging caltrin from the surface of spermatozoa. There are sufficient evidences in favour of this hypothesis (see Rufo et al. 1984, Ruknudin 1989, Babcock et al. 1990).

Sidhu & Guraya (1989b) proposed a model based upon their studies on buffalo spermatozoa. They isolated a calmodulin-like protein (CLP) from buffalo seminal plasma, using anti-CLP anti-

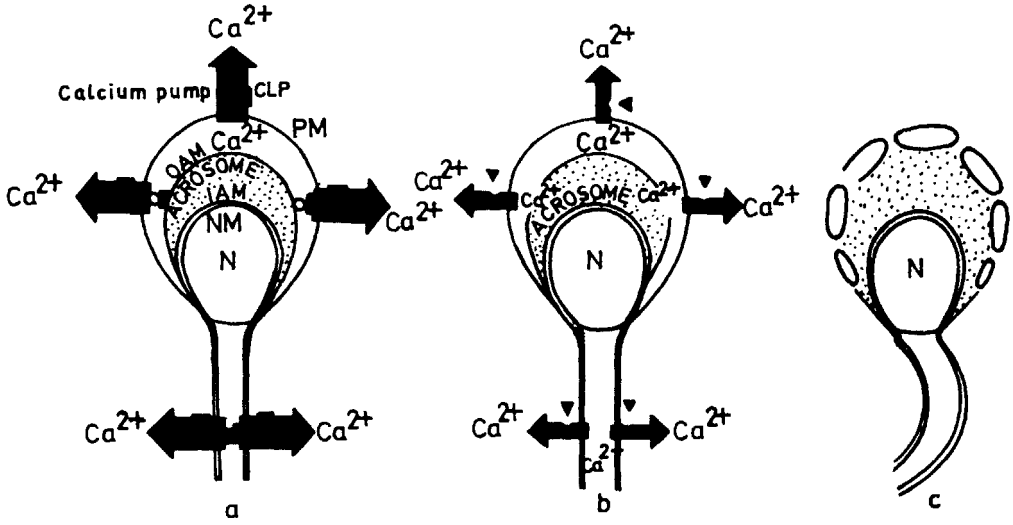

Figure 2 Model explaining regulation of Ca^{2+} in buffalo spermatozoa during ejaculation (figure 2a), capacitation (figure 2b) and acrosome reaction (figure 2c). Details of explanation in the text. CLP, calmodulin-like protein; IAM, inner acrosomal membrane; N, nucleus; NM, nuclear membrane; OAM, outer acrosomal membrane; PM, plasma membrane. From Sidhu and Guraya (unpublished observations)

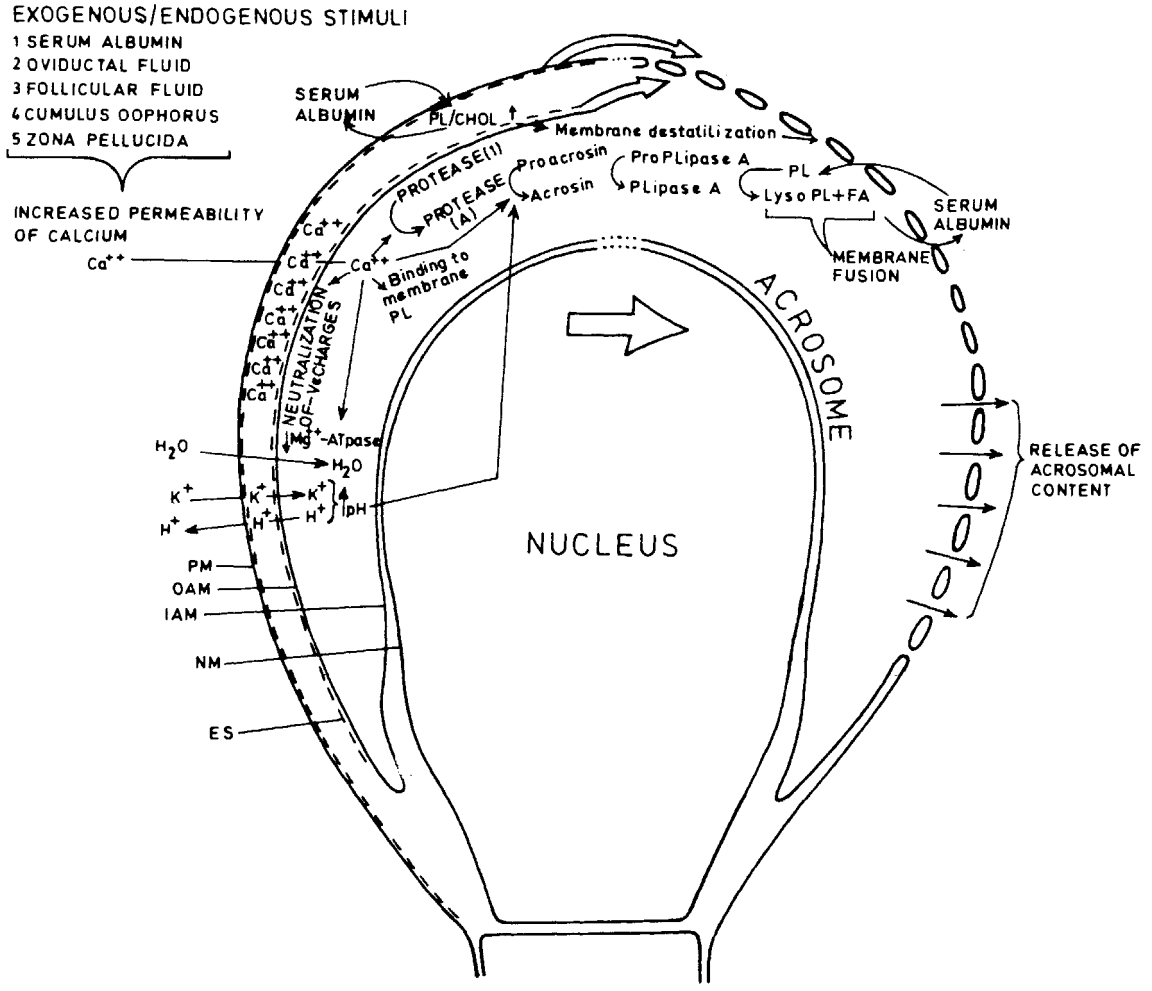

Figure 3 Hypothesis showing various changes observed during Ca^{2+}-induced AR in mammalian spermatozoa. Several exogenous and endogenous stimuli bring about membrane alterations during capacitation and probably facilitate Ca^{2+} influx. The increased intraacrosomal Ca^{2+} might bring about (1) membrane vesculation of plasma membrane and outer acrosomal membrane; (2) inhibition of Mg^{2+}-ATPase conserves intraacrosomal H_2O required for acrosomal swelling; (3) binding to acidic phospholipids (PL), forming crystalline domains in the membrane at the site of fusion; (4) activating a putative zymogen protease (I) (inhibited) to protease (A) (activated) required for activation of proacrosin to acrosin; (5) stabilizing acrosin activity, which might be involved directly for membrane vesiculation or might activate prophospholipase A_2 (Pro PLipase) producing fusogen lysophosphatides (Lyso PL) plus fatty acid (FA). The role of serum albumin might be to remove these FA thus preventing end-product inhibition of PLipase A, or it might be involved in chelating cholesterol, thus lowering the cholesterol (CHOL)/PL ratio (i.e., increasing the PL/CHOL ratio), leading to membrane destabilization. The increased intraacrosomal *p*H possibly brought about by the proton pump is necessary for activation of proacrosin to acrosin and also for PLipase activity. PM, plasma membrane; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane; NM, nuclear membrane; ES, equatorial segment. From Sidhu and Guraya (1989a)

PM, plasma membrane; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane; NM,

bodies and indirect immunofluorescent method, CLP has been localized on the surface of buffalo spermatozoa. CLP is present in sufficient quantity in buffalo seminal plasma and binds to the surface of spermatozoa during ejaculation. CLP greatly stimulates sperm plasma membrane bound Ca^{2+}, Mg^{2+}-ATPase (Sidhu & Guraya 1989b) and thus stimulates Ca^{2+} extrusion phenomenon (figure 2a). During capacitation it has been demonstrated by us that some of the sperm surface proteins are probably removed (Sidhu et al. 1984) suggesing the strong possibility of the removal of CLP. As shown in figure 2b, this will slow down the Ca^{2+} extrusion phenomenon in sperm consequently leading to accumulation of intracellular Ca^{2+} in spermatozoa that brings about membrane vesiculation during AR (figure 2c). Peterson et al. (1983), however, proposed that Ca^{2+} influx rather Ca^{2+} efflux is regulated by calmodulin in boar spermatozoa.

In all the hypotheses explained, there is a general consensus that there is a rapid influx of Ca^{2+} by some mechanism immediately before the AR, which is essential for bringing about vasiculation of plasma membrane and outer acrosomal membrane and that leads to the release of acrosomal contents required for fertilization. However, the mechanism by which Ca^{2+} induces AR is not clear. Based on the available literature and our own findings on AR, we propose a hypothesis explaining mechanism of the action of the intracellular Ca^{2+} for AR in mammalian spermatozoa (Sidhu & Guraya 1989a). For explanation, see figure 3 and its legend. The concepts described in this figure are based on the available data and might need to be revised in future with further advances made on this subject (Sidhu & Guraya 1989a).

In summary it can be concluded that the rapid influx of Ca^{2+} in spermatozoa occurs during capacitation and during the onset of AR (Peterson et al. 1983, Fraser 1987, Thomas & Meizel 1988, Stock & Fraser 1989, Ruknudin 1989, White & Aitken 1989, Cox & Peterson 1989). The mechanism by which rapid influx of Ca^{2+} is brought about during AR in spermatozoa is far from clear. The involvement of sperm ATPases, Ca^{2+} channels, Na^+/Ca^{2+}-antiporter, etc. has been proposed. Only recently the purified membranes from mammalian spermatozoa have been used to study the mechanism of Ca^{2+} transport (Rufo et al. 1984, Cox & Peterson 1989, Sidhu & Guraya 1989b, Breitbart et al. 1990). Though these *in vitro* studies using sperm membrane vesicles have demonstrated the involvement of ATPases, Ca^{2+} channels and Na^+/Ca^{2+} antiporter in transporting Ca^{2+}, but these vesicles may have lost the regulatory features found in the intact cells. Many exogenous substances like phorbos ester (for elucidating the role of diacylglycerol), ionophore (for elucidating the role of inositol triphosphate), calmodulin, calmodulin like protein, caltrin/seminal plasmin have been used for defining their role in Ca^{2+} transport. Many conflicting findings are reported in literature. No unified view has emerged which can explain ubiquitously the mechanism of Ca^{2+} transport in sperm from different species. But this subject will continue to be interesting and the future studies will reveal all the controversies that exist in literature regarding regulation of Ca^{2+} transport in spermatozoa.

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