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Regulatory mechanisms in acrosomal exocytosis

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Acrosomal exocytosis occurs after the binding of the spermatozoon to the zona pellucida of the oocyte via specific receptors. We suggest that the zona pellucida binds to at least two different receptors in the plasma membrane. One (R) is a G_i-coupled receptor that activates phospholipase $C\beta_1$. The other (TK) is a tyrosine kinase receptor coupled to phospholipase Cγ. Binding to R would regulate adenylyl cyclase leading to an increase in cyclic adenosine monophosphate and protein kinase A activation. The protein kinase A activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane that releases Ca^{2+} from the interior of the acrosome to the cytosol. This is the first (I), relatively small, rise in intracellular Ca^{2+} which leads to activation of the phospholipase Cγ. The products of phosphatidyl-inositol bisphosphate hydrolysis by phospholipase C, diacylglycerol and inositol-trisphosphate lead to protein kinase C translocation to the plasma membrane and its activation. Protein kinase C opens a voltage-dependent Ca^{2+} channel (L) in the plasma membrane, leading to the second (II), higher, increase in intracellular Ca^{2+} leading to acrosomal exocytosis. Spermine, a physiological constituent of the seminal plasma regulates sperm acrosomal exocytosis by modulating intracellular Ca^{2+} binding sites and phospholipase C activity. Spermine is rapidly incorporated into the sperm cells during ejaculation and temporarily inhibits premature capacitation and acrosome reaction. During the passage of the spermatozoon through the female genital tract, there is a progressive depletion of spermine from spermatozoa, so that capacitation and consequently the acrosomal exocytosis take place at the appropriate time, when the spermatozoon reaches the vicinity of the egg.

Intact spermatozoa bind to the zona pellucida via specific receptors that are localized over the anterior head region of the spermatozoon. Binding to the zona pellucida stimulates the spermatozoon to undergo the acrosome reaction (Kopf and Gerton, 1991). This is a stimulus–secretion coupled exocytotic event in which the exocytotic vesicle (the acrosome) fuses with the overlying plasma membrane (reviewed in Yanagimachi, 1994; Brucker and Lipford, 1995). The multiple fusions between the outer acrosomal membrane and the plasma membrane result in the release of hydrolytic enzymes (mostly acrosin) and in the exposure of new membrane domains, both of which are essential if fertilization is to proceed further. The hydrolytic enzymes released from the acrosome digest the zona pellucida, allowing the spermatozoon to approach the egg and fertilize it. Acrosome reaction will follow zona pellucida binding only if the spermatozoon has previously undergone a poorly defined process of maturation known as capacitation. Capacitation occurs *in vivo* upon exposure of the spermatozoon to the female reproductive tract, but can be induced *in vitro* in the presence of various synthetic media. There is no clear recognizable marker for the occurrence of capacitation. However, several intracellular changes are known to occur, including increases in membrane fluidity, protein tyrosine phosphorylation, and cAMP concentrations, decreases in the cholesterol:phospholipid ratio of the plasma membrane and net surface charge, and changes in swimming patterns and chemotactic motility (Cohen-Dayag *et al.*, 1995).

Progesterone has been implicated as being another natural ligand that induces acrosome reaction (Melendrez *et al.*, 1994; Meyers *et al.*, 1995). The effects of progesterone on the generation of intracellular messengers, such as diacylglycerol (DAG), are mimicked by γ-aminobutyric acid (GABA), suggesting that progesterone acts on a sperm $GABA_A$ receptor. This receptor is a unique steroid receptor–Cl^- channel complex resembling, but not identical to, the $GABA_A$ receptor–Cl^- channel from mammalian central nervous system neurones (Wistrom and Meizel, 1993). Meizel and coworkers have suggested the involvement of a glycine receptor–Cl^- channel (GlyR) (Melendrez and Meizel, 1995) and have recently identified and localized the GlyR in the head of pig spermatozoa (Melendrez and Meizel, 1996).

Progesterone appears to act in synergy with the zona pellucida during the physiological acrosome reaction (Melendrez *et al.*, 1994) and this interaction may provide important insights into the mechanism of the reaction by identifying the convergence points of different signalling pathways.

Acrosome reaction is induced *in vitro* in capacitated spermatozoa by incubation with solubilized zona pellucida. The ensuing signal transduction cascades invoke a host of enzymatic activities and other effects (Table 1), and progress of the acrosome reaction is accompanied by an increase in the cytosolic calcium concentration followed by an increase in pH (reviewed in Florman *et al.*, 1990). Alkalization is an important component in human spermatozoa (Brook *et al.*, 1966) and ZP3-induced acrosome reaction (Arnoult *et al.*, 1996), whereas it is probably less important for the progesterone-induced reaction. (Hamanah *et al.*, 1996). Acrosome reaction can also be induced *in vitro* by ionophores such as A23187 that exchange Ca^{2+} for $2H^+$. More

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Table 1. Factors involved in acrosome reaction

Factor	Possible functions in acrosome reaction	Calcium dependence for activation	Involved in membrane fusion
Tyrosine kinases	Transmembrane signalling Phosphorylation of PLCγ Activation of a Na^+/H^+ exchanger Activation of L-type Ca^{2+} channels	Submicromolar	No*
G-proteins	Activation of adenylyl cyclase Activation of $PLC\beta_1$ H^+ efflux	Submicromolar	No
Adenylyl cyclase/cAMP/PKA	Release of calcium from acrosomal stores	Submicromolar	No
PKC	Opening of plasma membrane calcium channels Activation of PLA_2	Micromolar	No
PIP_2 specific PLC/IP_3	Enhancement of membrane fusibility Activation of PKC, PLA_2 Actin depolymerization Release of Ca^{2+} from acrosomal stores	Micromolar	Yes
PLA_2/arachidonic acid	Enhancement of membrane fusibility Calcium entry Activation of PKC	Micromolar	Not determined
Actin-severing proteins/actin depolymerization	Removal of the F-actin barrier to fusion	Supramicromolar	Yes
Membrane proteins	Membrane fusion	Supramicromolar	Yes

The factors listed have been implicated as being essential components of acrosome reaction since their inhibition prevents the reaction, and they have been localized in the periacrosomal region of the sperm head. Since the calcium concentration in the cell is submicromolar before the onset of the reaction, factors maximally active at this calcium concentration are essentially calcium independent. The partition of the other factors into two groups on the basis of their calcium requirement for maximal activation (micromolar or supramicromolar), suggests a mechanism of acrosome reaction that involves an increase in calcium concentration at the fusion sites which occurs in two stages. The same inhibitors used to show the involvement of these factors in acrosome reaction were also used to determine which factors are invoked specifically in the membrane fusion stage of the reaction using the cell-free assay of membrane fusion. The function of factors involved in acrosome reaction, but not in membrane fusion, is probably to regulate the calcium concentration at the fusion sites.

PK, protein kinase; PL, phospholipase; PIP_2, phosphatidylinositol 4, 5-bisphosphate; IP_3, inositol 1, 4, 5-trisphosphate.

*Membrane fusion in a cell free system is inhibited by phosphatase treatment, indicating the importance of protein phosphorylation for the fusion process (Spungin *et al.*, 1995).

recently, thapsigargin has also been shown to increase cytoplasmic Ca^{2+} concentrations and induce the acrosome reaction (Meizel and Turner, 1993a; Spungin and Breitbart, 1996) in capacitated spermatozoa. Thapsigargin was originally described as a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump (Thastrup *et al.*, 1990) and its effects in spermatozoa were the first indication that intracellular calcium ($[Ca^{2+}]_i$) might be recruited during the reaction. In the case of ionophore- or thapsigargin-induced acrosome reaction, parts of the signal transduction cascades are bypassed, but the resulting acrosome reaction appears to be morphologically indistinguishable from that induced by the zona pellucida.

The selection of Ca^{2+} as an intracellular messenger requires precise regulation of its concentration. In considering the mechanisms involved in $[Ca^{2+}]_i$ regulation, we should discriminate between spontaneous and ligand-induced events. The sperm systems involved in $[Ca^{2+}]_i$ regulation include the plasma membrane, $[Ca^{2+}]_i$ stores, probably the acrosome, and the mitochondria. The plasma membrane contains a Na^+–Ca^{2+} exchanger (Rufo *et al.*, 1984), an ATP-dependent Ca^{2+} pump (Breitbart *et al.*, 1983) and a voltage-sensitive Ca^{2+} channel (Babcock and Pfeiffer, 1987). According to Florman's model, interaction of ZP3 with the sperm receptor activates a poorly selective cation channel, leading to membrane depolarization and a G-protein (G_i) dependent pH_i regulation leading to intracellular alkalization. These two changes control the opening of the voltage-sensitive Ca^{2+} channel (Florman *et al.*, 1995). The entry of Ca^{2+} via this channel, resulting in an increase in $[Ca^{2+}]_i$ will stimulate the $3Na^+$–Ca^{2+} exchanger and the Ca^{2+} pump to excrete Ca^{2+} from the cell. Thus, the voltage-sensitive Ca^{2+} channel is characterized as a ligand-induced event, whereas the other two systems may be considered as spontaneous events.

During progesterone initiation of the human sperm acrosome reaction, there is a several-fold increase in $[Ca^{2+}]_i$, although the type of Ca^{2+} channel involved is not yet known (Blackmore *et al.*, 1990). Absence of Na^+ inhibits the progesterone mediated $[Ca^{2+}]_i$ increase (Garcia and Meizel, 1994), and it has been suggested that Na^+ is required for pH_i regulation (Garcia and Meizel, 1996).

Role of polyamines

The synthesis of polyamines may be necessary for the increase in $[Ca^{2+}]_i$ caused by progesterone (Meizel and Turner, 1993b).

The polyamine spermine at 1 mmol l^{-1} enhances by twofold Ca^{2+} uptake by the sperm cells; however, there is a concentration-dependent reversal of this stimulation when the polyamine concentration is increased to 5 or 10 mmol l^{-1} (Breitbart and Rubinstein, 1995). This high concentration of spermine also causes a 50% inhibition in Ca^{2+} uptake or acrosomal exocytosis induced by the Ca^{2+}-ionophore, A23187 (Breitbart and Rubinstein, 1995). A very low concentration of spermine (10 μmol l^{-1}) induced acrosomal exocytosis, whereas millimolar concentrations inhibited this effect (Rubinstein *et al.,* 1995). Kinetic studies revealed the presence of high (kDa = 3.6×10^{-5}) and low (kDa = 1.2×10^{-3}) affinity spermine binding sites in ram spermatozoa (Rubinstein and Breitbart, 1991). Cytochemical methods revealed that most of the polyamines were localized in the midpiece, where the sperm mitochondria are located, and in the acrosome region (Rubinstein and Breitbart, 1994). Ultrastructural methods revealed that the outer acrosomal membrane is the preferred site for spermine binding, which was densely distributed, and there was only a sparse distribution of binding sites on the plasma membrane surrounding the acrosome and none on the postacrosomal region (Rubinstein and Breitbart, 1994). Spermine is a polycation and, therefore, anionic sites may function as binding sites. Anionic sites have been identified in the acrosome and midpiece regions (Yanagimachi *et al.,* 1972).

It was suggested that the anionic sites on the acrosome region are the binding sites for Ca^{2+} that promote fusion between the outer acrosomal membrane and the overlying plasma membrane during acrosomal exocytosis. Indeed, Ca^{2+} was detected on the outer acrosomal membrane of several mammalian spermatozoa (Plummer and Watson, 1985; Berruti *et al.,* 1986; Ruknudin, 1986) and Ca^{2+} is necessary for inducing fusion between isolated outer acrosomal and plasma membranes in a cell free system (Spungin *et al.,* 1992).

Spermine plays a role in membrane fusion, which is part of the acrosomal exocytosis. Therefore, spermine binding sites on the outer acrosomal membrane and the plasma membrane surrounding the acrosome may play a crucial role in acrosomal exocytosis. Micromolar concentrations of spermine can induce fusion between isolated outer acrosomal and plasma membranes *in vitro* (Spungin *et al.,* 1992), in agreement with other data in which micromolar concentrations of spermine induced the occurrence of acrosomal exocytosis in intact cells (Rubinstein *et al.,* 1995).

Spermine can regulate the fluxes of Ca^{2+} to and from mitochondria isolated from rat liver or brain (Kroner, 1988). The presence of spermine binding sites surrounding the sperm mitochondria (Rubinstein and Breitbart, 1994) suggest that these sites may be involved in the regulation of Ca^{2+} in the mitochondria. We found that spermine enhances Ca^{2+} uptake into spermatozoa when the mitochondria are active in accumulating Ca^{2+} but inhibits Ca^{2+} uptake when the mitochondria are inactive (Breitbart and Rubinstein, 1995). When the sperm mitochondria are active in the presence of added mitochondrial substrates, 90% of the Ca^{2+} taken up is accumulated in the mitochondria, but in the absence of added substrate, Ca^{2+} is accumulated in non-mitochondrial regions of the cells (Breitbart and Rubinstein, 1995).

Calcium uptake into extramitochondrial regions and acrosomal exocytosis are both inhibited by 10 mmol spermine l^{-1} but there is no effect on $[Ca^{2+}]_i$ (Breitbart and Rubinstein, 1995). These data indicate that the positively charged spermine is transported into the cell and competes for negatively charged extramitochondrial Ca^{2+}-binding sites. These sites are probably involved in acrosomal exocytosis. As mentioned before, micromolar concentrations of spermine stimulate acrosomal exocytosis in intact cells as well as fusion of isolated membrane, while millimolar concentrations of spermine inhibit these two processes. In addition, high concentrations of spermine inhibit phospholipase C activity, which is essential for acrosomal exocytosis and for membrane fusion *in vitro* (Spungin and Breitbart, 1996).

In conclusion, spermine, a physiological constituent of the seminal plasma, regulates sperm acrosomal exocytosis by modulating intracellular Ca^{2+}-binding sites and phospholipase C activity. Our results indicate that spermine binds reversibly to spermatozoa by electrostatic interactions (Rubinstein and Breitbart, 1991). The rates of both binding and release in spermatozoa are very fast compared with other types of cell. The release of bound spermine is further facilitated by heparin, a constituent of the female reproductive tract, which induces capacitation and the acrosome reaction (Parrish *et al.,* 1988). We suggest that in species that have millimolar concentrations of spermine in the seminal plasma, spermine is incorporated rapidly into the spermatozoa during ejaculation, temporarily inhibiting premature capacitation and acrosome reaction. A progressive depletion of spermine from spermatozoa then occurs during the passage through the female genital tract, enhanced by heparin, so that sperm capacitation, and consequently the acrosome reaction, take place at the appropriate time, when the spermatozoon reaches the vicinity of the oocyte.

Receptor tyrosine kinase

Three proteins have been suggested as ZP3 receptor molecules on intact mouse spermatozoa (reviewed by Snell and White, 1996): a 56 kDa protein (Bleil and Wassarman, 1990); a β-1,4 galactosyl transferase (Miller *et al.,* 1992); and a tyrosine phosphorylated protein p95 (RTK) (Leyton and Saling, 1989). The RTK may activate a sperm Na^+–H^+-exchanger, promoting cell alkalization, membrane depolarization and activation of an L-like Ca^{2+}-channel (Fraser and McIntyre, 1989). In addition, a 95 kDa protein that is tyrosine-phosphorylated, and in which tyrosine-phosphorylation increases with capacitation, is present in human spermatozoa (Burks *et al.,* 1995). However, there has been some controversy over this work (Bork, 1996; Saling *et al.,* 1996; Tsai and Silver, 1996). Kopf's laboratory has identified a 95 kDa phosphotyrosine-containing protein in mouse spermatozoa which displays the properties of hexokinase (Kalab *et al.,* 1994). Although monoclonal antibody against p95 and anti-hexokinase antibody do not appear to recognize the same proteins (Leyton *et al.,* 1995), the roles of these two 95 kDa proteins in sperm–egg interaction remain to be clarified. Progesterone stimulates tyrosine phosphorylation of a 94 kDa human sperm protein (Tesarik *et al.,* 1993) which may be the 95 kDa receptor, RTK. Tyrosine kinase is also involved in progesterone-induced Ca^{2+} fluxes and the acrosome reaction (Tesarik *et al.,* 1996).

A receptor for epidermal growth factor (EGFR), which is also a tyrosine kinase and involved in the acrosome reaction,

has been identified in the head of bovine spermatozoa (Lax *et al.*, 1994; Breitbart *et al*., 1995a). This receptor may prove to be more significant during capacitation, since high concentrations of EGF are present in the female reproductive tract. During bovine sperm capacitation, there is an increase in tyrosine phosphorylation of a 170 kDa and a 140 kDa protein which may be EGFR and PLCγ (phospholipase Cγ), respectively (Breitbart *et al*., 1995), and a significant increase in PLCγ binding to the plasma membrane after its tyrosine phosphorylation (Spungin *et al.*, 1995). In mouse spermatozoa, ZP3 activation of PLC-γ is mediated by tyrosine phosphorylation (Tomes *et al.*, 1996). Protein tyrosine phosphorylation stimulated by cAMP/protein kinase A (PKA) during capacitation takes place in mouse (Visconti *et al.*, 1995) and bull (Galantino-Homer *et al.*, 1995) spermatozoa.

Guanosine triphosphate (GTP) binding (G) proteins

Sperm cells contain two pertussis toxin substrates (G_i proteins) with molecular masses of 41 kDa and properties similar to those of somatic cell pertussis-sensitive G proteins (Kopf *et al.*, 1986). Pertusis toxin inhibits zona pellucida – but not ionophore-induced acrosome reaction (Endo *et al.*, 1987), showing that G_i-like proteins are involved upstream of the increases in Ca^{2+} concentration. Zona pellucida binding has also been shown to activate a membrane bound GTP-binding protein (Ward *et al.*, 1992). A causal connection between a G_i protein and adenylyl cyclase, as is found in somatic cells, has not yet been demonstrated in spermatozoa. Florman *et al*. (1995) suggested a model in which sperm binding to the zona pellucida activates a poorly selective cation channel, causing a membrane depolarization and a G_i protein-dependent H^+ efflux (intracellular alkalization). It is not known whether there is an interaction between the receptor tyrosine kinase and sperm G proteins. Cross-linking of galactosyl-transferase by specific antibodies activates G proteins and induces the acrosome reaction (Gong *et al.*, 1995).

Gαq/11 which activates $PLC\beta_1$ was recently identified in the acrosomal region of mammalian spermatozoa (Walensky and Snyder, 1995). A G protein of the subtype G_o has been located in the equatorial segment of the sperm head (Breitbart *et al*., 1995a). Its function has not been determined, but in other cells, G_o is involved in Ca^{2+} transport across the plasma membrane (Hescheler *et al.*, 1987). Cholera toxin sensitive proteins (G_s proteins) have not been found in spermatozoa (Kopf *et al.*, 1986).

Adenylyl cyclase/cAMP/protein kinase A

Intracellular concentrations of cAMP are increased during the acrosome reaction (Hyne and Garbers, 1979), indicating the activation of adenylyl cyclase. Inhibitors of cAMP-dependent protein kinase (PKA) have been shown to inhibit the acrosome reaction (De Jonge *et al.*, 1991). The membrane-bound adenylyl cyclase can be stimulated by the zona pellucida (Leclerc and Kopf, 1995). The adenylyl cyclase is regulated by protein phosphorylation, possibly via a tyrosine kinase (Kurvari *et al.*, 1996). The role of cAMP in the acrosome reaction may be to release Ca^{2+} from an acrosomal store that is partially inhibited by the PKA inhibitor H89 (Spungin and Breitbart, 1996). This suggests the presence on the acrosomal membrane of either a cAMP-gated Ca^{2+} channel (reviewed by Kaupp, 1991) or a channel opened upon phosphorylation by PKA (reviewed by Reuter, 1987). A Ca^{2+} channel opened by cAMP has been detected in sea urchin spermatozoa (Cook and Babcock, 1993), and a cyclic nucleotide-gated Ca^{2+} channel from mammalian spermatozoa has been cloned and functionally expressed in oocytes (Weyand *et al.*, 1994). This cAMP-dependent acrosomal channel is voltage-dependent since Ca^{2+} release via this channel is inhibited by nifedipine (Spungin and Breitbart, 1996). This is consistent with a large body of evidence showing the involvement of voltage-dependent calcium channels in the acrosome reaction (Fraser and McIntyre, 1989; Florman *et al.*, 1992). Walensky and Snyder (1995) suggest that rat sperm acrosome contains an ATP-dependent Ca^{2+} pump and IP_3-gated Ca^{2+} channel, indicating the possible involvement of the acrosome in intracellular Ca^{2+} regulation.

Protein kinase C

Protein kinase C inhibitors and stimulators have been used to show that PKC is involved in the acrosome reaction (Breitbart *et al.*, 1992; Rotem *et al.*, 1992). Western blot analysis showed that the PKC_α and $PKC_{\beta I}$ isoforms are localized to the plasma membrane in bovine spermatozoa (Lax *et al*., 1997). This translocation is very rapid (1 min), depends upon the presence of Ca^{2+} and is independent of sperm capacitation. We also identified the presence in the plasma membrane of RACK, the binding protein for PKC (Lax *et al*., 1997). Two possible roles for PKC in the acrosome reaction have been suggested. One possible role is to activate a plasma membrane Ca^{2+} channel (Spungin and Breitbart, 1996) to generate an essential increase in $[Ca^{2+}]_i$ (Babcock and Pfeiffer, 1987; Breitbart *et al.*, 1990), since the PKC-dependent plasma membrane calcium channel is voltage-dependent (Spungin and Breitbart, 1996). The second role suggested for PKC in the acrosome reaction is activation of phospholipase A_2 (PLA_2). Stimulation of PLA_2 generates arachidonic acid (Roldan *et al.*, 1992) which is further metabolized to prostaglandins (PG) and leukotrienes by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX), respectively. $PGF_{2\alpha}$ production during the acrosome reaction is inhibited by the PKC inhibitor staurosporin, and this inhibition is overcome by exogenous arachidonic acid (Breitbart *et al*., 1995b).

Phosphatidylinositol bisphosphate-specific phospholipase C_γ/inositol trisphosphate (IP_3)

During capacitation, actin polymerization leads to the rapid formation of actin filaments bound to the plasma and outer acrosomal membranes. This is followed by a slower attachment of PLCγ, presumably after its phosphorylation, to this membrane bound actin (Spungin *et al.*, 1995). Inhibition of this PLCγ by neomycin inhibits the acrosome reaction. Moreover, acrosome reaction is restored in the presence of neomycin when an exogenous, neomycin-insensitive, PIP_2 -specific PLC of bacterial origin is added (Spungin and Breitbart, 1996). We have suggested several roles for this membrane bound PLC. The diacylglycerol it produces would enhance the fusibility of the membranes (Siegel *et al.*, 1989) and activate PKC and PLA_2. Another effect of this PLC would be to hydrolyse phosphatidylinositol phosphate (PIP) and PIP_2 bound to actin severing proteins, thereby alleviating the PIP_2 inhibition of these

proteins (Janmey, 1994). PLCβ_1 has been identified in the acrosomal region of mammalian spermatozoa, and IP_3 has been shown to induce release of Ca^{2+} from the acrosome (Walensky and Snyder, 1995). It was also shown that progesterone induces the production of DAG and acrosome reaction in human spermatozoa (O'Toole *et al.*, 1996).

Phospholipase A_2/arachidonic acid

Studies by several laboratories (Llanos *et al.*, 1982; Lax *et al.*, 1990) have shown that the hydrolysis products of PLA_2 activity on membrane phospholipids (lysophospholipids and arachidonic acid) are involved in the acrosome reaction. Inhibitors of PLA_2 and LOX inhibit the acrosome reaction while the products of these enzymes, arachidonic acid and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HETE), stimulate the acrosome reaction in capacitated spermatozoa (Joyce *et al.*, 1987; Lax *et al.*, 1990; Breitbart *et al*., 1995b). However, Mack *et al.* (1992) have suggested that the human sperm acrosome reaction does not depend on arachidonic acid metabolism, although previously they had suggested a role for COX and prostaglandins in the guinea-pig sperm acrosome reaction (Joyce *et al.*, 1987). However, even their recent data show that NDGA or indomethacin inhibit the acrosome reaction induced by A23187 or dibutyryl cAMP, respectively, indicating that LOX and COX are in fact involved in the human sperm acrosome reaction.

In bovine spermatozoa, COX is localized mainly in the sperm head (Shalev *et al.*, 1994). Exogenous PGE_2 enhances Ca^{2+} uptake (Shalev *et al.*, 1994) and stimulates the acrosome reaction which is completely inhibited by the LOX inhibitor NDGA, indicating that the LOX pathway is involved in the mechanism by which the COX pathway stimulates the acrosome reaction (Breitbart *et al*., 1995b). Acrosome reaction induced by exogenous arachidonic acid is inhibited by NDGA but not by the COX inhibitor indomethacin (Lax *et al.*, 1990). (NDGA can inhibit PLA_2 as well, but this is irrelevant when the acrosome reaction is induced by exogenous arachidonic acid.) Prostaglandin E_2 also stimulates the production of $PGF_{2\alpha}$. Acrosome reaction and $PGF_{2\alpha}$ production stimulated by PGE_2, are both inhibited by the PLA_2 inhibitors, quinacrin and dibromoacetophenone, or by the PKC inhibitor staurosporin, which is overridden in the presence of exogenous arachidonic acid (Breitbart *et al*., 1995b). These results showed the involvement of PGE_2 in the activation of PLA_2 via PKC in a positive feedback loop, releasing more arachidonic acid for 15-HETE synthesis, which is necessary for the acrosome reaction (Chang *et al.*, 1987; Lax *et al.*, 1990).

It is not clear how this positive feedback loop is first activated. It has been proposed that diacylglycerol generated from the hydrolysis of polyphosphoinositides by PLC could activate PLA_2 directly during the acrosome reaction of ram spermatozoa (Roldan and Harrison, 1989). In hamster spermatozoa, entry of Ca^{2+} into the cells activates PLA_2 (Imai *et al.*, 1990). It is possible that prostaglandins present in the seminal plasma cause the first activation of PLA_2, as relatively high concentrations are found in human semen (Bygdemen and Samuelsson, 1966). Prostaglandin E_2 stimulates Ca^{2+} uptake by the cells, possibly via PKC, which would activate PLA_2 (Shalev *et al.*, 1994). In addition, $PGF_{2\alpha}$ inhibits Ca^{2+} uptake by bovine spermatozoa (Shalev *et al.*, 1994). The antagonism between the two prostaglandins may be significant for the regulation of Ca^{2+} entry into the cells. These results are in agreement with others showing that PGE_2, but not $PGF_{2\alpha}$, induced a sustained increase in human sperm penetration rates (Aitken and Kelly, 1985).

When COX is inhibited by indomethacin, there is probably enough free arachidonic acid in the cell to operate the LOX pathway so that the acrosome reaction would not be inhibited. When COX is active, there is a greater need in the cell for free arachidonic acid for the COX and LOX pathways. Under these conditions, the product of the COX pathway, PGE_2, can further stimulate PLA_2 to release more arachidonic acid. When PLA_2 is inhibited, free arachidonic acid can be released by the sequential action of phospholipase C and diacylglycerol lipase (Chang *et al.*, 1987). Although the COX pathway does not play a direct role in the mechanism of acrosome reaction, it may have an important role in other sperm functions such as motility (Aitken and Kelly, 1985). Thus, the spermatozoon can synthesize PGE_2 for these functions, and at the same time keep the LOX pathway going by supplying arachidonic acid via activation of the PLA_2 by PGE_2.

Microtubule-associated protein kinase

Our data indicate the presence of MAPK (42 kDa) in sperm cells (Fig. 1). It is known that activated MAPK phosphorylates a large number of regulatory proteins and can directly control cellular processes including cytoskeletal rearrangement. We showed (Spungin *et al.*, 1995; Spungin and Breitbart, 1996) that F-actin rearrangement is involved in sperm capacitation and acrosome reaction. Activation of epidermal growth factor receptor (EGFR) by EGF stimulates the translocation and activation of PLCγ, which precedes F-actin binding to the plasma membrane (Spungin *et al.*, 1995). We hypothesized that the MAPK cascades are involved in the regulation of F-actin rearrangement by RTK.

Actin severing proteins/actin depolymerization

The F-actin network between the plasma and outer acrosomal membranes forms a scaffolding holding the PLCγ that is involved in acrosome reaction at the membrane surface. However, dispersion of this F-actin network between the two membranes is necessary for the acrosome reaction since inhibition of actin depolymerization by phalloidin inhibits the reaction (Spungin *et al.*, 1995). The observation that both actin depolymerization (Spungin and Breitbart, 1996) and membrane fusion (Spungin *et al.*, 1995) require supramicromolar calcium supports the notion that the actin filaments constitute the final barrier to fusion (Table 1)

We have studied the acrosome reaction using a cell-free system in which the membrane fusion event is isolated from other stages of the signal transaction cascades (Spungin *et al.*, 1992; 1995; Spungin and Breitbart, 1996).

The cell-free fusion assay was used to determine which of the factors known to be involved in the acrosome reaction in intact cells are required specifically for the membrane fusion stage of the reaction (Table 1). The effects of inhibitors of the various factors on cell-free fusion were examined. Of the factors tested, only the activity of a PIP_2-specific PLC and the depolymerization of membrane bound actin were found to be essential for membrane fusion (Table 1).

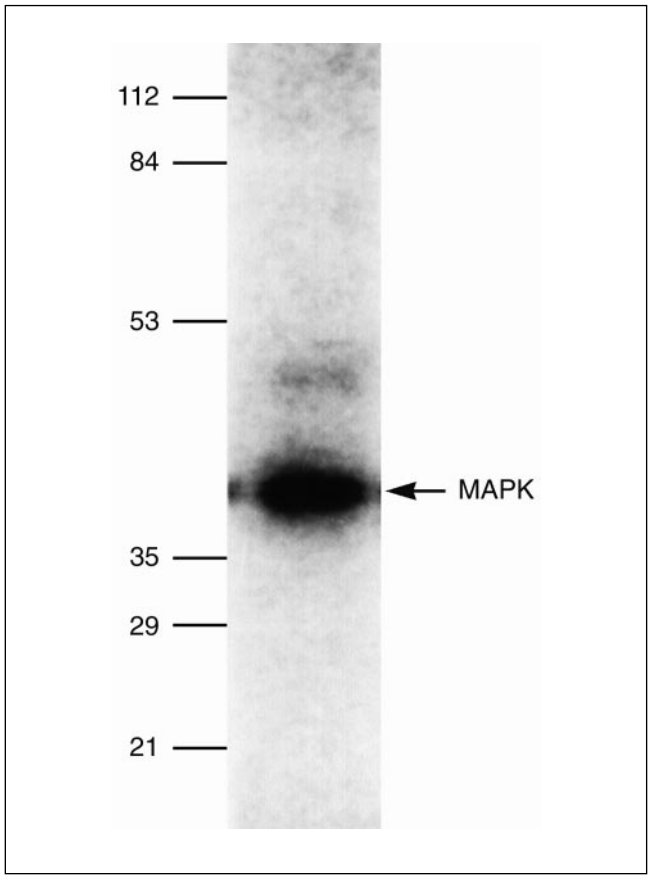

Fig. 1. Detection of microtubule-associated protein kinase (MAPK; 42 kDa) in bovine spermatozoa after SDS-PAGE and blotting, using anti-MAPK.

Roles of calcium

The acrosome reaction involves at least four Ca^{2+}-requiring enzyme activities: (1) PIP_2-specific PLC (Roldan and Harrison, 1989; Spungin *et al.*, 1995); (2) protein kinase C (Breitbart *et al.*, 1992); (3) PLA_2 (Roldan *et al.*, 1992); and (4) actin depolymerization (Spungin *et al.*, 1995; Spungin and Breitbart, 1996). These activities vary in their Ca^{2+} requirement for activation (Table 1).

The difference in the Ca^{2+} requirement for PLC activation (micromolar) and membrane fusion (supramicromolar) suggests that these two parameters could serve as reporters of Ca^{2+} concentration at the fusion sites during the acrosome reaction. The acrosome reaction in ram spermatozoa, for example, has been shown to comprise two stages. The first stage involves the PIP_2-specific PLC activity which is complete within 3 min. Membrane fusion occurs only several minutes later (Roldan and Harrison, 1989). Similar observations have been made in human spermatozoa (Thomas and Meizel, 1989). Before the onset of acrosome reaction, the Ca^{2+} concentration at the fusion sites is submicromolar (Table 1). During the first 3 min of the acrosome reaction, when PLC activity, but not fusion, is occurring, the Ca^{2+} concentration at the fusion sites is micromolar, and this subsequently rises to supramicromolar concentrations for the membrane fusion stage of the reaction. This would contradict conclusions, based on observations of concentrations of $[Ca^{2+}]_i$ using indicators such as fura-2, that Ca^{2+} concentrations remain submicromolar during the reaction (Florman *et al.*, 1989; Bailey and Storey, 1994). However, these indicators give a spatially averaged Ca^{2+} concentration which may differ considerably from the concentration at the fusion sites (Janmey, 1994). The fusion sites for exocytosis are positioned close to clusters of plasma membrane Ca^{2+} channels, and only after influx via these channels does the concentration of Ca^{2+} immediately local to the cytosolic aspect of the channels increase sufficiently to support membrane fusion. At these sites, the concentration of Ca^{2+} has been theoretically calculated to be as high as 100 μmol l^{-1} (See Sihra *et al.*, 1992), which corresponds to the concentration needed for actin release (Spungin and Breitbart, 1996) or membrane fusion (Spungin *et al.*, 1995) during the acrosome reaction.

Work with the cell-free system identified factors involved in the acrosome reaction in intact cells but not in the membrane fusion step of the reaction. Our working hypothesis was that the function of these factors in the acrosome reaction is regulating the concentration of Ca^{2+} at the fusion sites since all Ca^{2+} regulatory mechanisms are overridden in the cell-free fusion assay. We tested this hypothesis by studying Ca^{2+} uptake and release into extracted plasma membrane vesicles and extracted intact acrosomes. The results showed that sperm plasma membranes possess a thapsigargin-insensitive Ca^{2+} pump and Ca^{2+} channels that are opened by phosphorylation by PKC. The acrosomal membrane was found to possess a Ca^{2+} pump that is inhibited by thapsigargin, and Ca^{2+} channels that are opened by cAMP (Spungin and Breitbart, 1996). This finding suggests that there is either a cAMP-gated Ca^{2+} channel or a channel opened upon phosphorylation by PKA. The observation that thapsigargin induces the acrosome reaction and inhibits the acrosome Ca^{2+} pump (Meizel and Turner, 1993; Spungin and Breitbart, 1996) suggests that the acrosome serves as a store of Ca^{2+} that is mobilized during the acrosome reaction. Calcium deposits have been detected in the acrosome (Berruti and Franchi, 1986) and calreticulin, an endoplasmic reticulum sequestering protein, has been detected by immunocytochemistry in mouse acrosomes (Nakamura *et al.*, 1992).

Conclusions

The PLC activity essential for membrane fusion is associated with membrane-bound F-actin but is also involved in its depolymerization (via actin severing proteins). Disassembly of this F-actin would thus have to be delayed until the PLC has completed its role in the acrosome reaction. The theoretical need to separate in time the activation of PLC and depolymerization of the F-actin, which require different Ca^{2+} concentrations for maximal effect (Table 1), is consistent with a model of the acrosome reaction involving an increase in free Ca^{2+} at the fusion sites that occurs in two stages (Fig. 2). An increase in $[Ca^{2+}]_i$ occurring in two stages has been observed during progesterone-induced acrosome reaction in human spermatozoa (Tesarik *et al.*, 1996). Progesterone also induces a Ca^{2+}-dependent increase in cAMP (Parinaud and Milhet, 1996).

Binding of solubilized zona pellucida to extracted sperm membranes has been shown to activate a membrane-bound adenylyl cyclase in a Ca^{2+}-independent fashion (Schackmann and Chock, 1986; Leclerc and Kopf, 1995). Therefore, this

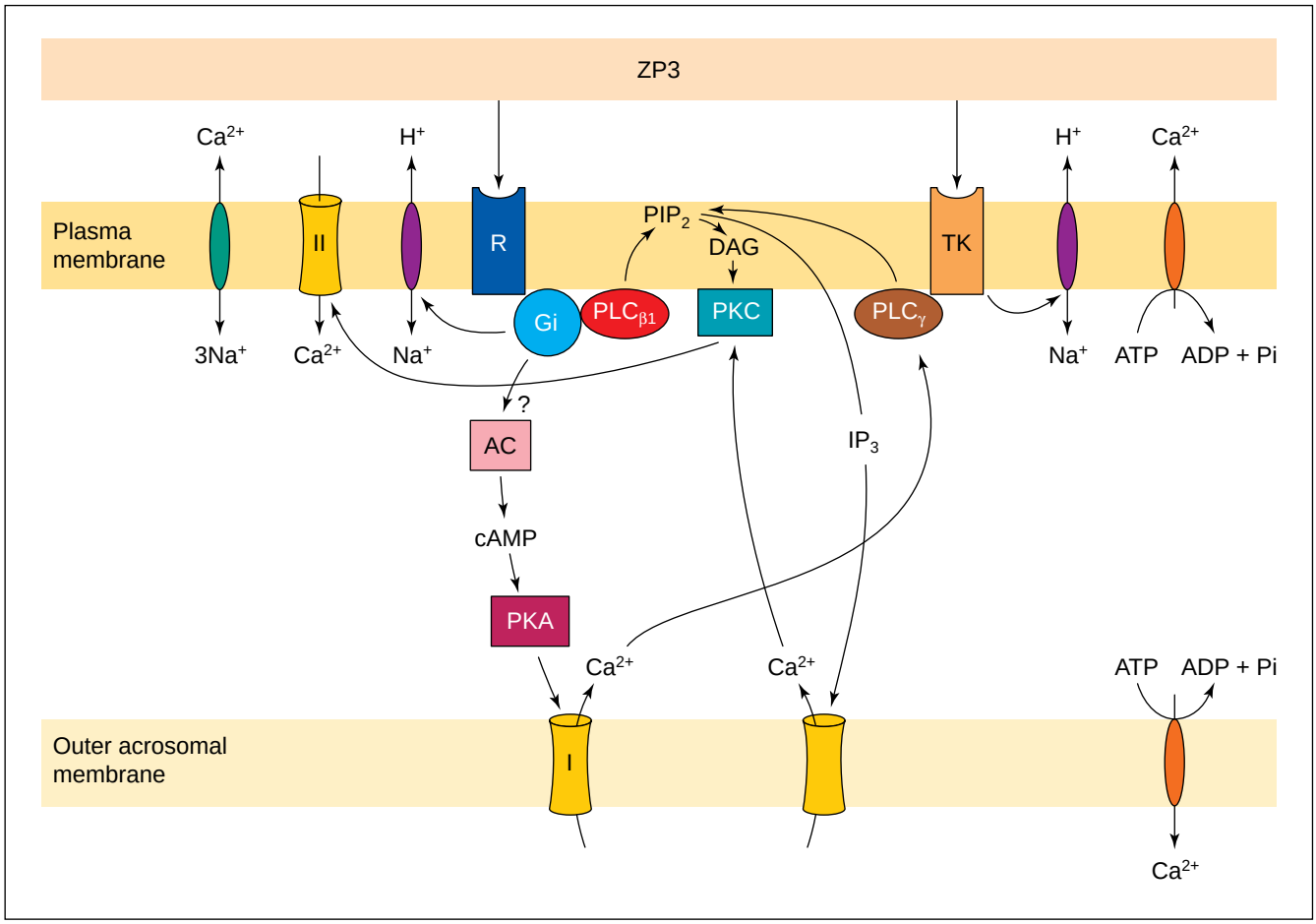

Fig. 2. Possible interactions among the activities invoked during the acrosome reaction. The glycoprotein, ZP3, binds to at least two different receptors in the plasma membrane. One (R) is a G_i coupled receptor that activates phospholipase $C\beta_1$ ($PLC\beta_1$). The other (TK) is a tyrosine kinase receptor coupled to PLCγ. Binding to R would regulate adenylate cyclase (AC) leading to increased cAMP and protein kinase A (PKA) activation. The PKA activates a voltage dependent Ca^{2+} channel in the outer acrosomal membrane which releases Ca^{2+} from the interior of the acrosome to the cytosol. This is the first (I), relatively small, rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) which leads to activation of the PLCγ. The products of phosphatidylinositol 4, 5-bisphosphate (PIP_2) hydrolysis by PLC diacylglycerol (DAG) and inositol-triphosphate (IP_3) will lead to PKC translocation to the plasma membrane and its activation. Protein kinase C opens a voltage dependent Ca^{2+} channel (L) in the plasma membrane, leading to the second (II), higher, increase in $[Ca^{2+}]_i$. The G_i or TK can also activate a Na^+/H^+ exchanger, leading to alkalization of the cytosol. The increase in $[Ca^{2+}]_i$ and pH will lead to membrane fusion and acrosomal exocytosis.

represents an early event in the signal transduction pathway. Activation of this adenylyl cyclase would increase cAMP concentrations which, in turn, would open the acrosomal Ca^{2+} channels. This mobilization of Ca^{2+} would generate the first increase in Ca^{2+} concentration at the fusion sites (from submicromolar to micromolar calcium), activating the membrane-bound PLC. Consistent with this, PLC activity in intact cells has been shown to be inhibited by voltage-dependent Ca^{2+} channel blockers (Harrison *et al.*, 1990). This increase in Ca^{2+} concentration and activation of PLC also occurs in response to progesterone (Roldan *et al.*, 1994). This PIP_2-specific PLC activity would have several effects. The conversion of PIP_2 to diacylglycerol would enhance the fusibility of the membranes. This PLC will also remove the PIP_2-inhibition of actin-severing proteins. The diacylglycerol produced could also activate other phospholipases, such as PLA_2 and phosphatidylcholine-specific PLC, which would further prime the membranes for fusion. Finally, the diacylglycerol would activate PKC which would open the plasma membrane Ca^{2+} channels. The resulting influx of Ca^{2+} would generate the second increase in Ca^{2+} concentration at the fusion sites (to supramicromolar concentrations). This would activate the actin severing proteins leading to the dispersion of the F-actin barrier intervening between the outer acrosomal and overlying plasma membranes. The primed membranes would then be able to come into contact and fuse, releasing the acrosomal contents, and completing the acrosome reaction.

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