

The biochemistry of the acrosome reaction*

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The binding of the spermatozoon to the oocyte zona pellucida (ZP) occurs via specific receptors localized over the anterior head region of the spermatozoon. Zona pellucida binding stimulates the spermatozoa to undergo the acrosome reaction resulting in the release of hydrolytic enzymes and in the exposure of new membrane domains, both of which are essential for fertilization. We suggest that ZP binds to at least two different receptors in the plasma membrane. One (R) is a G_i-coupled receptor that activates phospholipase C (PLC) β_1 . The other (TK) is a tyrosine kinase receptor coupled to PLC γ . Binding to R would regulate adenylyl cyclase (AC) leading to elevation of cAMP and protein kinase (PKA) activation. The PKA activates a voltage-dependent Ca²⁺ channel in the outer acrosomal membrane which releases Ca²⁺ from the interior of the acrosome to the cytosol. This is the first, relatively small, rise in [Ca²⁺]_i (I) which leads to activation of the PLC γ . The products of phosphatidyl-inositol bisphosphate (PIP₂) hydrolysis by PLC diacylglycerol (DAG) and inositol-trisphosphate (IP₃) will lead to PKC translocation to the plasma membrane and its activation. PKC opens a voltage-dependent Ca²⁺ channel (L) in the plasma membrane, leading to the second (II) higher increase in [Ca²⁺]_i. The G_i or TK can also activate an Na⁺/H⁺ exchanger leading to alkalization of the cytosol. PKC also activates phospholipase A₂ (PLA₂) to generate arachidonic acid (AA) from membrane phospholipids. AA will be converted to prostaglandins (PG) and leukotriens (LT) by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) respectively. The increase in [Ca²⁺]_i and pH leads to membrane fusion and acrosomal exocytosis.

Key words: acrosome reaction/hydrolytic enzymes/intracellular calcium concentration/membrane domains/plasma membrane receptors

Introduction

After penetrating the cumulus oophorus of the ovum, the spermatozoon binds to the zona pellucida with its plasma membrane intact. Sperm binding to the zona pellucida occurs via specific receptors to a zona pellucida glycoprotein (ZP3 in the mouse) which are localized over the anterior head region of the spermatozoa. Zona pellucida binding stimulates the spermatozoa 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 spermatozoa to approach the egg and fertilize it. Acrosome reaction will follow zona pellucida binding only if the spermatozoa has previously undergone a poorly defined process of maturation known as capacitation. Capacitation occurs *in vivo* upon exposure of the spermatozoa

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.

Progesterone has been implicated as being another natural ligand which induces acrosome reaction (Osman *et al.*, 1989; Meizel *et al.*, 1990; Melendrez *et al.*, 1994; Roldan *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 neurons (Wistrom and Meizel, 1993; Sabeur *et al.*, 1996). Meizel and coworkers have suggested the involvement of a glycine receptor/Cl⁻ channel (GlyR) (Melendrez and Meizel, 1995; Turner and Meizel, 1995) and recently they identified and localized the GlyR in the head of porcine spermatozoa (Melendrez and Meizel, 1996). Progesterone seems to act in

*Presented at the Sperm Biochemistry Symposium.

Table I. Factors involved in the acrosome reaction

Factor	Possible functions in acrosome reaction	Calcium dependence for activation	Involved in membrane fusion
Tyrosine kinases	Transmembrane signalling Phosphorylation of PLC γ Activation of a Na ⁺ /H ⁺ exchanger Activation of L-type Ca ²⁺ channels	submicromolar	No
G-proteins	Activation of adenylyl cyclase Activation of PLC β_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

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 prior to the onset of the reaction, factors which are 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 involving an elevation of calcium concentration at the fusion sites which occurs in two stages. The same inhibitors which were used to show the involvement of these factors in acrosome reaction were also used to determine which of them 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. See text for references and abbreviations.

synergy with zona pellucida in physiological acrosome reaction (Melendrez *et al.*, 1994; Roldan *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.

In-vitro acrosome reaction in intact cells

Acrosome reaction is induced *in vitro* in capacitated spermatozoa by incubation with solubilized zona pellucida. The ensuing signal transduction cascade invokes a host of enzymatic activities and other effects (Table I), and progress of the acrosome reaction is accompanied by an elevation in the cytosolic calcium concentration followed by an elevation in pH (reviewed in Florman *et al.*, 1990). Alkalization is an important component in human spermatozoa (Brook *et al.*, 1996) 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 which exchange Ca²⁺ for 2H⁺. More recently, thapsigargin has also been shown to raise cytoplasmic Ca²⁺ levels (Blackmore, 1993; Serres *et al.*, 1994) and induce acrosome reaction (Meizel and Turner, 1993; Spungin and Breitbart, 1996) in capacitated spermatozoa. Thapsigargin was originally described as a specific inhibitor of the endoplasmic reticulum Ca²⁺ pump (Thastrup *et al.*, 1990). Its effects in spermatozoa were the first indication that intracellular calcium 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 zona pellucida.

Receptor tyrosine kinase

Leyton and Saling (1989) found a 95 kDa receptor protein for ZP3, which has the structure of a receptor tyrosine kinase (RTK). This receptor has been suggested to activate a sperm Na⁺/H⁺-exchanger which promotes cell alkalization, membrane depolarization and activation of a Ca²⁺-channel similar to the dihydropyridine-sensitive Ca²⁺-channel which predominates in muscle cells (Fraser, 1993). A 95 kDa protein that is tyrosine-phosphorylated is present in human spermatozoa and its level of tyrosine-phosphorylation increases with capacitation (Burks *et al.*, 1995). Recently, 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 with properties of hexokinase (Kaleb *et al.*, 1994). It is not known whether there is an interaction between the receptor tyrosine kinase and sperm G-proteins (Ward and Kopf, 1993). Progesterone stimulates tyrosine phosphorylation of a 94 kDa human sperm protein (Tesarik *et al.*, 1993) which may be the 95 kDa receptor RTK.

A receptor for epidermal growth factor (EGFR), which is also a tyrosine kinase and involved in acrosome reaction, was identified in the head of bovine spermatozoa (Lax *et al.*, 1994;

Breitbart *et al.*, 1995). This receptor may actually be more significant during capacitation, since high amounts 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 PLC γ (phospholipase C γ) respectively (Breitbart *et al.*, 1995), and a significant increase in PLC γ binding to the plasma membrane after its tyrosine phosphorylation (Spungin *et al.*, 1995). Protein tyrosine phosphorylation stimulated by cAMP/protein kinase A (PKA) during capacitation was recently shown to take place in mouse (Visconti *et al.*, 1995) and bull (Galantino-Homer *et al.*, 1995) spermatozoa.

GTP binding (G)-proteins

Detergent extracts of invertebrate and vertebrate spermatozoa contain two pertussis toxin substrates (G_i proteins) with molecular masses of ~41 000 kDa and properties similar to those of somatic cell pertussis-sensitive G-proteins (Kopf *et al.*, 1986). Pertussis toxin inhibits zona pellucida but not ionophore-induced acrosome reaction (Endo *et al.*, 1987), showing that G_i-like proteins are involved upstream to Ca²⁺ elevation. 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 (Fraser, 1993). Florman *et al.* (1995) have 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).

G α q/11 was recently identified in the acrosomal region of mammalian spermatozoa which activates PLC β ₁ (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.*, 1995). Its function has not been determined but, in other cells, G_o is involved in Ca²⁺ transport across the plasma membrane (Hescheler *et al.*, 1987). Cholera toxin-sensitive proteins (G_s proteins) have not been found in spermatozoa (Hildebrandt *et al.*, 1985; Kopf *et al.*, 1986).

Adenylyl cyclase/cAMP/PKA

Intracellular levels of cAMP are elevated during acrosome reaction (Hyne and Garbers, 1979; Ward and Kopf, 1993), indicating the activation of the enzyme adenylyl cyclase, and inhibitors of cAMP-dependent protein kinase (PKA) have been shown to inhibit acrosome reaction (De Jonge *et al.*, 1991a). The membrane bound adenylyl cyclase can be stimulated by the zona pellucida (Leclerc and Kopf, 1995). The role of cAMP in acrosome reaction may be release of calcium from an acrosomal store which is partially inhibited by the PKA inhibitor H89 (Spungin and Breitbart, 1996). This suggested the existence on the acrosomal membrane of either a cAMP-gated calcium channel (reviewed in Kaupp, 1991) or a channel opened upon phosphorylation by PKA (reviewed in Reuter, 1987). A calcium channel opened by cAMP has been detected in sea urchin spermatozoa (Cook and Babcock, 1993), and a cyclic nucleotide-gated calcium 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 calcium 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 acrosome reaction (Fraser and McIntyre, 1989; Cox *et al.*, 1991; Florman *et al.*, 1992).

Protein kinase C (PKC)

PKC inhibitors and stimulators have been used to show that PKC is involved in acrosome reaction (De Jonge *et al.*, 1991b; Breitbart *et al.*, 1992; Rotem *et al.*, 1992). Western blot analysis showed that the PKC α isoform is localized to the plasma membrane in bovine spermatozoa (Breitbart *et al.*, 1995). Two possible roles for PKC in acrosome reaction have been suggested. One is to activate a plasma membrane calcium channel (Galizzi *et al.*, 1987; Spungin and Breitbart, 1996) to generate an essential increase in intracellular Ca²⁺ (Babcock and Pfeiffer, 1987; Breitbart *et al.*, 1990). The PKC-dependent plasma membrane calcium channel is voltage-dependent (Spungin and Breitbart, 1996). PKC activated calcium channels have also been observed in other cell types (Nastainczyk *et al.*, 1987; O'Callahan *et al.*, 1988; Ma *et al.*, 1992; Bode and Goke, 1994; Schultzmman and Groscher, 1994; Baranska *et al.*, 1995).

Another role suggested for PKC in acrosome reaction is activation of phospholipase A₂ (PLA₂). Stimulation of PLA₂ generates arachidonic acid (Roldan *et al.*, 1992) which is further metabolized to prostaglandins (PGs) and leukotriens by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) respectively. PGF_{2 α} production during acrosome reaction is inhibited by the PKC inhibitor staurosporin, and this inhibition is overcome by exogenous arachidonic acid (Breitbart *et al.*, 1995). PKC has also been shown to enhance arachidonic acid release in other cell types (Slivka and Insel, 1988; Halenda *et al.*, 1989; Murayama *et al.*, 1990; Dempster *et al.*, 1992; Kozawa *et al.*, 1992; Liu *et al.*, 1992; Tokuda *et al.*, 1992).

Phosphatidyl-inositol bisphosphate (PIP₂)-specific PLC/inositol trisphosphate (IP₃)

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 PLC γ , presumably after its phosphorylation, to this membrane bound actin (Spungin *et al.*, 1995). Inhibition of this PLC γ by neomycin inhibits acrosome reaction. Moreover, acrosome reaction is restored in the presence of neomycin when an exogenous, neomycin insensitive, PIP₂-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; Luk *et al.*, 1993) and activate PKC and PLA₂. Another effect of this PLC would be to hydrolyse phosphatidyl-inositol phosphate (PIP) and PIP₂ bound to actin severing proteins thereby alleviating the PIP₂ inhibition of these proteins (Goldschmidt-Clermont *et al.*, 1991; Janmey, 1994). PLC β ₁ has been identified in the acrosomal region of mammalian spermatozoa, and IP₃ 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).

PLA₂/arachidonic acid

Studies by several laboratories (Llanos *et al.*, 1982; Ono *et al.*, 1982; Bennet *et al.*, 1987; Lax *et al.*, 1990) have shown that the hydrolysis products of PLA₂ activity on membrane phospholipids (lysophospholipids and arachidonic acid) are involved in acrosome reaction. Inhibitors of PLA₂ and LOX inhibit acrosome reaction while the products of these enzymes, arachidonic acid and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HETE) stimulate acrosome reaction in capacitated spermatozoa (Meizel and Turner, 1984; Joyce *et al.*, 1987; Lax *et al.*, 1990; Breitbart *et al.*, 1995). Recently, however, Mack *et al.* (1992) suggested that human sperm acrosome reaction does not depend on arachidonic acid metabolism, although previously they had suggested a role for COX and PGs in guinea-pig sperm acrosome reaction (Joyce *et al.*, 1987). However, even their recent data show that NDGA or indomethacin inhibit acrosome reaction induced by A23187 or dibutyl-cAMP respectively, indicating that LOX and COX are in fact involved in human sperm acrosome reaction.

In bovine spermatozoa, COX is localized mainly in the sperm head (Shalev *et al.*, 1994). Exogenous PGE₂ enhances Ca^{2+} uptake (Shalev *et al.*, 1994) and stimulates acrosome reaction which is completely inhibited by the LOX inhibitor NDGA, indicating that the LOX pathway is involved in the mechanism in which the COX pathway stimulates acrosome reaction (Breitbart *et al.*, 1995). 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 PLA₂ as well, but this is irrelevant when the acrosome reaction is induced by exogenous arachidonic acid.) PGE₂ was also found to stimulate the production of PGF_{2 α} . Acrosome reaction and PGF_{2 α} production stimulated by PGE₂, are both inhibited by the PLA₂ inhibitors quinacrin or dibromoacetophenone or by the PKC inhibitor staurosporin, which is overridden in the presence of exogenous arachidonic acid (Breitbart *et al.*, 1995). These results showed the involvement of PGE₂ in the activation of PLA₂ via PKC in a positive feedback loop, in order to release more arachidonic acid for 15-HETE synthesis which is necessary for 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 PLA₂ directly during acrosome reaction of ram spermatozoa (Roldan and Harrison, 1989). In hamster spermatozoa, entry of Ca^{2+} into the cells activates PLA₂ (Imai *et al.*, 1990). It is possible that prostaglandins present in the seminal plasma cause the first activation of PLA₂ since relatively high concentrations are found in human semen (Bygdemen and Samuelsson, 1966). PGE₂ stimulates Ca^{2+} uptake by the cells, possibly via PKC, which would activate PLA₂ (Shalev *et al.*, 1994). We also found that PGF_{2 α} inhibits Ca^{2+} uptake by bovine spermatozoa (Shalev *et al.*, 1994). The antagonism between

the two PGs might be significant for the regulation of Ca^{2+} entry into the cells. These results are in agreement with others who showed that PGE₂, but not PGF_{2 α} 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 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, PGE₂, can further stimulate PLA₂ to release more arachidonic acid. When PLA₂ 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 might have an important role in other sperm functions such as motility (Aitken and Kelly, 1985). Thus, the spermatozoon can synthesize PGE₂ for these functions, and at the same time keep the LOX pathway going by supplying arachidonic acid via activation of the PLA₂ by PGE₂.

Actin severing proteins/actin depolymerization

The F-actin network intervening between the plasma and outer acrosomal membranes forms a scaffolding holding the PLC γ which is involved in acrosome reaction at the membrane surface. However, dispersion of this F-actin intervening between the two membranes is necessary for 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 I).

Cell-free assay of acrosome reaction

We have studied acrosome reaction using a cell-free system in which the membrane fusion event is isolated from other stages of the signal transduction cascades (Spungin *et al.*, 1992, 1995; Spungin and Breitbart, 1996). In this cell-free system of sperm exocytosis, plasma and outer acrosomal membranes are extracted and purified from bovine spermatozoa and labelled with chlorophyll *a* and *N,N'*-diactadecyloxycarbocyanine-*p*-toluene sulphonate (DCY) respectively. Membrane fusion is assayed by monitoring the merging of lipid bilayers which is revealed by resonance energy transfer between the two membrane labels which occurs only when they are located on the same membrane. In a typical experiment (Figure 1), labelled plasma outer acrosomal membranes are mixed in the reaction cuvette, and incubated for 1 min at pH 6.8 and 4 μM CaCl_2 . For membranes extracted from capacitated cells, fusion occurs when the calcium concentration is raised to 200 μM and the pH simultaneously raised to 7.4. Resonance energy transfer is measured in a spectrophotometer by exciting the DCY at 486 nm, and observing the sensitized fluorescence of the chlorophyll *a* at 676 nm. Both membrane species must be from capacitated cells for successful fusion, and the require-

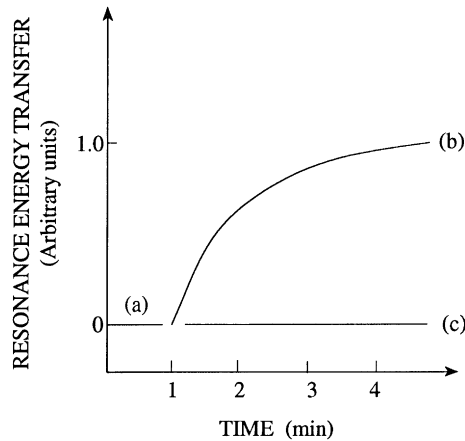


Figure 1. The cell-free assay of membrane fusion. In a typical experiment, acrosomal membranes are suspended in a pH 6.8 buffer containing $4 \mu\text{M Ca}^{2+}$. Under these conditions, fusion does not occur (curve a). After 1 min, the pH is raised to 7.4 by the addition of Tris-HCl, pH 10, and $200 \mu\text{M CaCl}_2$ is added simultaneously and fusion ensues (curve b). Fusion is indicated by the increase in resonance energy transfer which occurs only in the fusion product. Fusion does not occur if 1 mM neomycin [an inhibitor of phosphatidyl-inositol bisphosphate (PIP_2)-specific phospholipase C (PLC)] is present in the reaction medium (curve c), implicating this enzyme in the membrane fusion step of the reaction.

ments of pH and calcium mimic those of acrosome reaction in intact cells. The fusion was found to be highly specific (capacitated plasma and outer acrosomal membranes are unable to fuse with themselves). These observations showed that the membrane fusion which we observe in our system is not artefactual, but represents the physiological membrane fusion event that occurs during acrosome reaction.

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

Role of calcium

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

The difference in the calcium requirement for PLC activation (micromolar) and membrane fusion (supramicromolar) suggested that these two parameters could serve as reporters of calcium concentration at the fusion sites during acrosome reaction. Acrosome reaction in ram spermatozoa, for example, has been shown to be composed of two stages. The first involves the PIP_2 -specific $\text{PLC}\gamma$ activity which is complete within 3 min. Membrane fusion occurs only several minutes

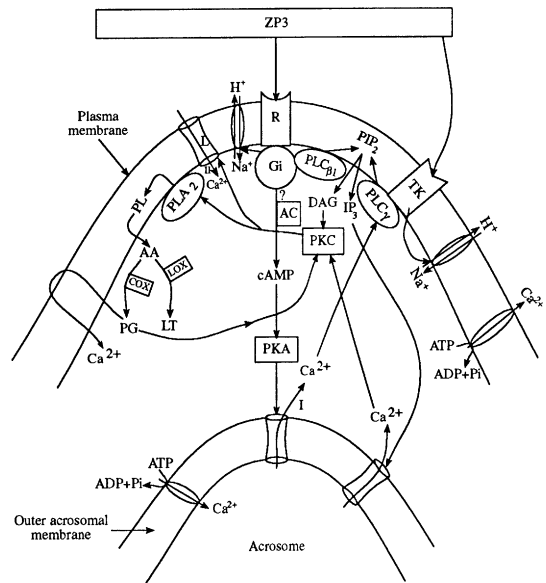


Figure 2. Possible interactions among the activities invoked during acrosome reaction. Zona pellucida glycoprotein (ZP3) binds to at least two different receptors in the plasma membrane. One (R) is a G_i -coupled receptor that activates phospholipase C ($\text{PLC}\beta_1$). The other (TK) is a tyrosine kinase receptor coupled to $\text{PLC}\gamma$. Binding to R would regulate adenylate cyclase (AC) leading to elevation of cAMP and protein kinase (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, relatively small, rise in $[\text{Ca}^{2+}]_i$ (I) which leads to activation of the $\text{PLC}\gamma$. The products of phosphatidyl-inositol bisphosphate (PIP_2) hydrolysis by PLC diacylglycerol (DAG) and inositol-trisphosphate (IP_3) will lead to protein kinase C (PKC) translocation to the plasma membrane and its activation. PKC opens a voltage-dependent Ca^{2+} channel (L) in the plasma membrane, leading to the second (II) higher increase in $[\text{Ca}^{2+}]_i$. The G_i or TK can also activate an Na^+/H^+ exchanger, leading to alkalization of the cytosol. PKC also activates phospholipase A_2 (PLA_2) to generate arachidonic acid (AA) from membrane phospholipids. AA will be converted to prostaglandins (PG) and leukotriens (LT) by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) respectively. The increase in $[\text{Ca}^{2+}]_i$ and pH will lead to membrane fusion and acrosomal exocytosis.

later (Roldan and Harrison, 1989). Similar observations have been made in human spermatozoa (Thomas and Meizel, 1989). We inferred from the data of Table I that prior to the onset of acrosome reaction, the calcium concentration at the fusion sites is submicromolar. During the first 3 min of the acrosome reaction, when PLC activity, but not fusion, is occurring the calcium concentration at the fusion sites is micromolar, and this subsequently rises to supramicromolar values for the membrane fusion stage of the reaction. This would contradict conclusions, based on observations of intracellular calcium concentrations using indicators such as FURA-2, that calcium concentrations remain submicromolar during the reaction (Florman *et al.*, 1989; Blackmore, 1993; Bailey and Storey, 1994; Serres *et al.*, 1994). However, these indicators give a spatially averaged calcium concentration which may be very different from the relevant concentration at the fusion sites (Janmey, 1994). This has been demonstrated in other cell types in which calcium influx (using A23187) or calcium mobilization (using thapsigargin) were used to raise the

intracellular calcium concentration. Although thapsigargin was at least as effective in raising the intracellular calcium concentration as calcium influx (as determined by FURA-2 measurements), only calcium influx was able to generate various physiological effects (Sihra *et al.*, 1992; Murayama *et al.*, 1993; Chiono *et al.*, 1995) including exocytosis in pancreatic cells (Murayama *et al.*, 1993). These latter observations were explained by the suggestion that the fusion sites for exocytosis are positioned close to clusters of plasma membrane calcium channels, and only after influx via these channels does the calcium concentration immediately local to the cytosolic aspect of the channels rise high enough to support membrane fusion. At these sites, the calcium concentration has been theoretically calculated to be as high as 100 μM (Sihra *et al.*, 1992), which corresponds to the calcium concentration needed for actin release (Spungin and Breitbart, 1996) or membrane fusion (Spungin *et al.*, 1995) during acrosome reaction.

Work with the cell-free system identified factors involved in 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 acrosome reaction is regulating the calcium concentration at the fusion sites since all calcium regulatory mechanisms are overridden in the cell-free fusion assay. We tested this hypothesis by studying calcium uptake and release into extracted plasma membrane vesicles and extracted intact acrosomes. The results showed that sperm plasma membranes possess a thapsigargin insensitive calcium pump and calcium channels which are opened by phosphorylation by PKC. The acrosomal membrane was found to possess a calcium pump which is inhibited by thapsigargin and calcium channels which are opened by cAMP (Spungin and Breitbart, 1996). This suggested the existence of either a cAMP-gated calcium channel or a channel opened upon phosphorylation by PKA. The observation that thapsigargin induces acrosome reaction and inhibits the acrosome calcium pump (Meizel and Turner, 1993; Spungin and Breitbart, 1996) suggests that the acrosome serves as a store of calcium which is mobilized during acrosome reaction. Calcium deposits have been detected in the acrosome (Berruti and Franchi, 1986; Watson and Plummer, 1986) and calreticulin, an endoplasmic reticulum sequestering protein has been detected by immunocytochemistry in mouse acrosomes (Nakamura *et al.*, 1992).

Conclusions

As mentioned above, the PLC activity which is essential for membrane fusion is attached to 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 roles in acrosome reaction. The theoretical need to separate in time the activation of PLC and depolymerization of the F-actin, which require different calcium concentrations for maximal effect (Table I), is consistent with a model of acrosome reaction involving an increase in free calcium at the fusion sites which occurs in two stages (Figure 2). An elevation in intracellular calcium occurring in two stages has been observed during progesterone induced acrosome reaction in human spermatozoa (Tesarik

et al., 1996). Progesterone also induces a calcium-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 calcium-independent fashion (Schackmann and Chock, 1986; Leclerc and Kopf, 1995). This would thus be an early event in the signal transduction pathway. Activation of this adenylyl cyclase would elevate cAMP concentrations which in turn would open the acrosomal calcium channels. This calcium mobilization would generate the first increase in calcium concentration at the fusion sites (from submicromolar to micromolar calcium; see above), activating the membrane bound PLC. Consistent with this, PLC activity in intact cells has been shown to be inhibited by voltage-dependent calcium channel blockers (Harrison *et al.*, 1990). This calcium elevation and activation of PLC also occurs in response to progesterone (Roldan *et al.*, 1994). This PIP₂-specific PLC activity would have several effects. The conversion of PIP₂ to diacylglycerol would enhance the fusibility of the membranes. This PLC will also remove the PIP₂-inhibition of actin severing proteins. The diacylglycerol produced could also activate other phospholipases such as PLA₂ and phosphatidylcholine specific PLC, which would further prime the membranes for fusion. Finally, the diacylglycerol would activate PKC which would open the plasma membrane calcium channels. The resulting calcium influx would generate the second increase in calcium concentration at the fusion sites (to supramicromolar values). 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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Received on July 30, 1996; accepted on December 12, 1996