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Protein kinases in mammalian sperm capacitation and the acrosome reaction

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Binding to the zona pellucida of an egg stimulates the spermatozoon to undergo the acrosome reaction, a process that enables it to penetrate the egg. Before this binding, the spermatozoon undergoes a series of biochemical transformations in the female reproductive tract, collectively called capacitation. Only capacitated spermatozoa can bind to the zona pellucida and undergo the acrosome reaction. Protein kinases may be involved in the regulation of intracellular Ca^{2+} during capacitation and the acrosome reaction. The first event in capacitation is the increase in intracellular calcium, bicarbonate and hydrogen peroxide, which collectively activate adenylyl cyclase to produce cyclic AMP, which activates protein kinase A to phosphorylate certain proteins. During capacitation, there is an increase in membrane-bound phospholipase C, and this binding is highly stimulated by the addition of epidermal growth factor to the cells. The capacitated spermatozoon binds to the zona pellucida of the egg via specific receptors and it is suggested that the zona pellucida binds to at least two different receptors in the sperm head plasma membrane. One is a G_i-coupled receptor that can activate phospholipase $C\beta_1$ and may regulate adenylyl cyclase to further increase cyclic AMP concentrations. The cyclic AMP activates protein kinase A to open a calcium channel in the outer acrosomal membrane, resulting in a relatively small increase in cytosolic calcium. This increase in Ca^{2+} leads to activation of phospholipase $C\gamma$, which is coupled to the second tyrosine kinase receptor. The products of phosphatidyl-inositol bisphosphate hydrolysis by phospholipase C, diacylglycerol and inositol-trisphosphate, induce the activation of protein kinase C and a calcium channel in the outer acrosomal membrane, respectively. Protein kinase C opens a calcium channel in the plasma membrane and, together with the inositol-trisphosphate-activated calcium channel, leads to a second and higher increase in cytosolic calcium. In addition, the depletion of calcium in the acrosome activates a capacitative calcium entry mechanism in the plasma membrane, leading to a rapid increase in cytosolic calcium (300–500 nmol l^{-1}). This increase in intracellular calcium concentration (and pH) leads to membrane fusion and the acrosome reaction.

Mammalian spermatozoa are unable to fertilize an egg immediately after ejaculation. They require a period of incubation in the female reproductive tract to acquire the capacity to fertilize. During this time, the spermatozoa undergo a poorly defined process of maturation known as capacitation. There is no clear recognizable marker for the occurrence of capacitation, although several intracellular changes are known to occur, including increases in membrane fluidity, cholesterol efflux, intracellular Ca^{2+} and cAMP concentrations, protein tyrosine phosphorylation, and changes in swimming patterns and chemotactic motility.

The head region of a capacitated spermatozoon expresses specific receptors that enable it to bind to the zona pellucida of an egg. Binding to the zona pellucida stimulates the spermatozoon to undergo the acrosome reaction, in which the outer acrosomal membrane fuses with the overlying plasma membrane (for review, see Yanagimachi, 1995; and Fig. 1). This exocytotic event results in the release of hydrolytic enzymes, principally the trypsin-like acrosin, and in the exposure of new membrane domains, both of which are essential for fertilization. The spermatozoon creates a groove approximately the width and height of the head to penetrate the zona pellucida (Allen and Green, 1997). It is still unclear how this groove is formed, but spermatozoa are incapable of forming it without having first undergone the acrosome reaction. The groove appears to be generated by a combination of physical pressure and enzymatic digestion of the zona pellucida (for review, see Gallicano *et al*., 1995).

The acrosome reaction can be induced *in vitro* in capacitated spermatozoa by incubation with solubilized zona pellucida or with ligands such as progesterone, epidermal growth factor, atrial natriuretic peptide or by $Ca^{2+}/2H^+$ ionophore A23187 (Breitbart *et al*., 1997). The ensuing signal transduction cascades invoke several protein kinases and an increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) followed by an increase in intracellular pH (for review, see Florman *et al*., 1998). This review provides an overview of the protein kinases involved in the signal transduction cascades responsible for the acrosome reaction.

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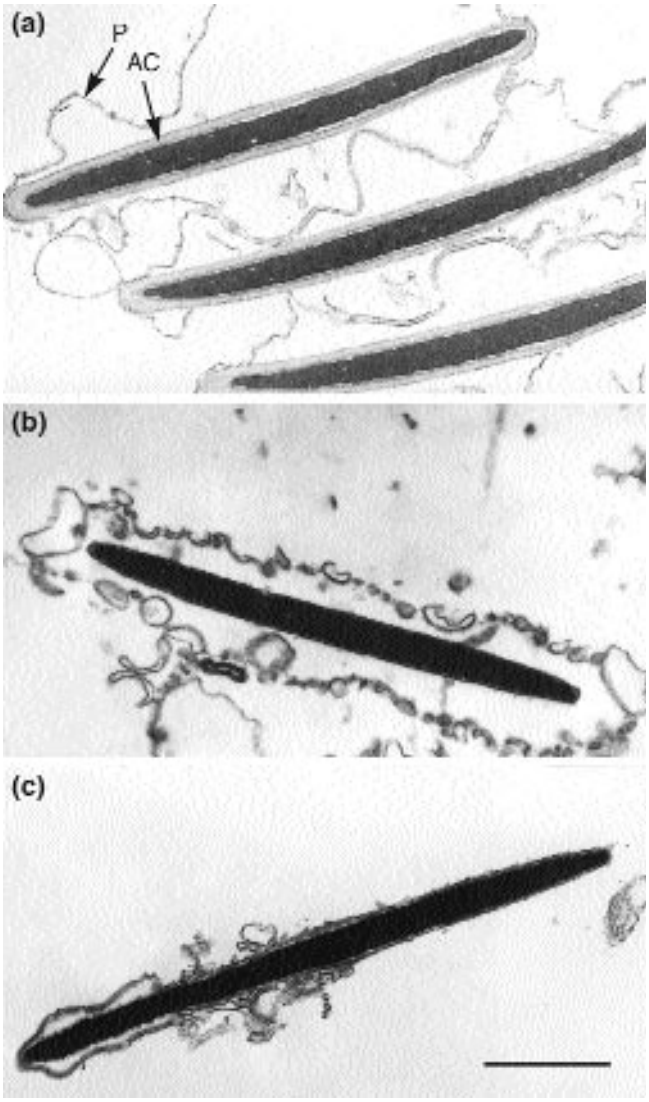

Fig. 1. Transmission electron micrographs of bovine sperm heads. (a) Intact spermatozoon. AC: acrosome; P: plasma membrane. (b) Acrosome reacted cell. Note the formation of mixed vesicles as a result of the fusion between the outer acrosomal membrane and the overlying plasma membrane. (c) Acrosome reacted cell. The mixed vesicles have disappeared, and the spermatozoon is now ready to penetrate the egg. Scale bar represents 1 μm.

Ca^{2+}–Mg^{2+}-ATPase: the ATP-dependent Ca^{2+} pump

The ATP-dependent Ca^{2+} pump (Ca^{2+}–Mg^{2+}-ATPase) is autophosphorylated by ATP and, therefore, can be classified as a protein kinase. It is hypothesized that the plasma membrane Ca^{2+} pump is involved in the regulation of $[Ca^{2+}]_i$ during sperm capacitation (Adeoya-Osiguwa and Fraser, 1993; DasGupta *et al*., 1994); however, as yet, there is no direct evidence to support this. The mechanism of $[Ca^{2+}]_i$ regulation within the spermatozoon involves the plasma membrane, intracellular Ca^{2+} stores, most likely in the acrosome, and the mitochondria. In the capacitated spermatozoon, the binding of the sperm receptor to the zona pellucida activates a poorly selective cation channel, leading to membrane depolarization and a G_i-protein-dependent increase in intracellular pH (pH_i), followed by the opening of a voltage sensitive Ca^{2+} channel and an increase in $[Ca^{2+}]_i$ (Florman *et al*., 1995).

In bull spermatozoa, $[Ca^{2+}]_i$ increases from 25 ± 10 to 160 ± 40 nmol l^{-1} during incubation under capacitating conditions (Table 1; Fig. 2). The entry of Ca^{2+} results in an increase in $[Ca^{2+}]_i$, which stimulates the ATP-dependent Ca^{2+} pump (Breitbart *et al*., 1983) and the plasma membrane $3Na^+$–Ca^{2+} exchanger (Rufo *et al*., 1984) to excrete Ca^{2+} from the cell. Non-capacitated spermatozoa may have decapacitating factors adsorbed on their membranes that activate a Ca^{2+}-ATPase to prevent an increase in $[Ca^{2+}]_i$ and premature capacitation. The loss of these decapacitating factors may cause a decrease in Ca^{2+}-ATPase activity, resulting in an increase in $[Ca^{2+}]_i$ (Adeoya-Osiguwa and Fraser, 1993). The decapacitating factors stimulate Ca^{2+}-ATPase activity in membranes isolated from the head, but not from the tail of mouse spermatozoa (Fraser, 1995; Adeoya-Osiguwa and Fraser, 1996). A decapacitating factor originating from the epididymis inhibits sperm capacitation and fertilizing capacity, but increases sperm motility, which is a hallmark of capacitated spermatozoa. This observation indicates that different mechanisms regulate $[Ca^{2+}]_i$ in the sperm head or tail (Fraser, 1995; Adeoya-Osiguwa and Fraser, 1996).

Although spermatozoa do not have endoplasmic reticulum, there is evidence that they possess intracellular Ca^{2+} stores. Treatment of capacitated spermatozoa with thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump, causes an increase in $[Ca^{2+}]_i$ (Blackmore, 1993) and induces the acrosome reaction (Meizel and Turner, 1993; Spungin and Breitbart, 1996) but only in the presence of extracellular Ca^{2+}. The Ca^{2+} from these stores may be recruited during the acrosome reaction. In support of this hypothesis, inositol trisphosphate receptor (IP_3R) is found in sperm acrosome and IP_3 induces the release of Ca^{2+} from intracellular Ca^{2+} stores (Walensky and Snyder, 1995) (Fig. 3). These findings indicate that the acrosome can serve as an intracellular Ca^{2+} store in spermatozoa. Active transport of Ca^{2+} into the acrosome may occur via an ATP-dependent Ca^{2+} pump, and the accumulated Ca^{2+} may be released by an IP_3-gated Ca^{2+} channel. When this Ca^{2+} pump is inhibited by thapsigargin, the concentration of Ca^{2+} in this store decreases, resulting in the opening of a capacitative Ca^{2+} entry mechanism in the plasma membrane (Putney, 1990). Thapsigargin induces an increase in $[Ca^{2+}]_i$ and the acrosome reaction only when extracellular Ca^{2+} is present, indicating that the opening of the capacitative Ca^{2+} entry mechanism occurs under these conditions (Spungin and Breitbart, 1996).

Studies of isolated bovine sperm acrosomes show that they possess an ATP-dependent Ca^{2+} pump that is inhibited by thapsigargin and Ca^{2+} channels that are opened by cAMP (Spungin and Breitbart, 1996). These data indicate the presence of either a cAMP-gated Ca^{2+} channel or a channel opened upon phosphorylation by cAMP-dependent protein kinase A (PKA). The observation that thapsigargin induces an increase in $[Ca^{2+}]_i$ and the acrosome reaction while inhibiting the acrosome Ca^{2+} pump indicates that the acrosome serves as a calcium store. Since cAMP concentrations and PKA-dependent protein tyrosine phosphorylation are enhanced during capacitation, and

Table 1. Intracellular $[Ca^{2+}]_i$ (nmol l^{-1}) in bovine spermatozoa before and after capacitation

Spermatozoa	Before adding Ca^{2+}	After adding Ca^{2+}	Thapsigargin before adding Ca^{2+}	Thapsigargin after adding Ca^{2+}
Uncapacitated	25 ± 10	35 ± 15	34 ± 16	500 ± 150
Capacitated	60 ± 20	160 ± 40	160 ± 40	1100 ± 250

Ejaculated bovine sperm (0.5×10^7 sperm ml^{-1}) were capacitated for 3 h with 2 mmol Ca^{2+} l^{-1} before adding 5 μmol Fura 2/AM l^{-1} for a further 1 h. At this time, the cells were washed, medium without Ca^{2+} was added for $[Ca^{2+}]_i$ determination before adding Ca^{2+}, and 2 mmol Ca^{2+} l^{-1} was added for further determination of $[Ca^{2+}]$. Changes in the fluorescence of the washed cells were determined at 340–380 nm excitation and 510 nm emission.
The values are means ± SEM of $[Ca^{2+}]_i$.

PKA can activate Ca^{2+} channels in the acrosomal membrane, it is suggested that the acrosomal calcium is mobilized during capacitation (Fig. 2).

Protein kinase A

The role of cAMP–PKA in capacitation and acrosome reaction remains uncertain. Sperm cAMP concentrations increase during capacitation in several mammalian species (White and Aitken, 1989; Parrish *et al*., 1994). Moreover, the activity of the cAMP-dependent protein kinase A (PKA) increases during mouse sperm capacitation, indicating a role for the cAMP–PKA signalling pathway in capacitation (Visconti *et al*., 1997). Inhibitors of PKA block the acrosome reaction, indicating a role for PKA in this reaction (De Jonge *et al*., 1991a). Sperm membrane-bound adenylyl cyclase activity (Leclerc and Kopf, 1995) and cAMP production are stimulated after binding to the zona pellucida. These observations indicate the activation of sperm adenylyl cyclase and an increase in cAMP after binding to the egg. Nevertheless, it is unclear how adenylyl cyclase is activated during capacitation, and why there is a requirement for a second activation of adenylyl cyclase after sperm binding to the zona pellucida but before the acrosome reaction. During the initial stages of capacitation, there is an increase in $[Ca^{2+}]_i$ and the production of reactive oxygen species (ROS, mainly H_2O_2) resulting in the activation of adenylyl cyclase. In sea urchin spermatozoa, cAMP is increased by hyperpolarization (Beltran *et al*., 1996). The role of cAMP/PKA in capacitation may be to indirectly regulate protein tyrosine phosphorylation (Visconti *et al*., 1995; Aitken *et al*., 1995; Leclerc *et al*., 1997), while its role in acrosome reaction may be to release Ca^{2+} from the acrosome (Spungin and Breitbart, 1996).

The acrosome reaction induced by dibutyryl-cAMP via activation of PKA in capacitated bovine spermatozoa is not inhibited by tyrosine kinase inhibitors (Table 2). Therefore, tyrosine phosphorylation is not involved in this stage of the acrosome reaction. Consistent with this view, the inhibition of PKC under these conditions does not affect the acrosome reaction (Table 2), indicating that stimulation of acrosome reaction by PKA is not dependent on PKC activity.

Immunocytochemical labelling reveals that bovine sperm RIα and RIβ subunits of PKA are both localized predominantly in the acrosomal segment of the head (Vijayaraghavan *et al*., 1997). These findings are consistent with the presence of PKA-dependent Ca^{2+} channels in the acrosome (Spungin and Breitbart, 1996). Although this Ca^{2+} channel may operate during the acrosome reaction (Breitbart and Spungin, 1997), it is possible that this activation is initiated by the increase in cAMP–PKA activity during capacitation.

Protein tyrosine kinase

Tyrosine phosphorylation of proteins plays an important role in capacitation (for example, see Visconti and Kopf, 1998), the acrosome reaction (e.g Burks *et al*., 1995) and sperm–zona penetration (for example, see Burks *et al*., 1995). Inhibition of tyrosine kinase by genistein blocks capacitation (Aitken *et al*., 1995). Omission of extracellular Ca^{2+} blocks capacitation but tyrosine phosphorylation still occurs, indicating that Ca^{2+} is involved in other events of capacitation (Luconi *et al*., 1996). Two different inhibitors of PKA that inhibit this enzyme by distinct mechanisms (Takagi *et al*., 1992) inhibit both protein tyrosine phosphorylation and capacitation of spermatozoa (Visconti *et al*., 1995). It is not clear whether the increase in tyrosine phosphorylation is due to the stimulation of tyrosine kinase, or to the inhibition of phosphotyrosine phosphatase, or to both mechanisms. In human spermatozoa, ROS upregulate the protein tyrosine phosphorylation of several proteins (Aitken *et al*., 1995; Leclerc *et al*., 1997) and, in mouse spermatozoa, H_2O_2 increases serine phosphorylation of a 80 kDa protein (H. Breitbart, unpublished). These observations are consistent with the suggestion that H_2O_2 activates adenylyl cyclase to produce cAMP and the subsequent activation of the serine–threonine kinase A (Aitken, 1997). Ca^{2+} and HCO_3^- may also be required for the activation of sperm adenylyl cyclase (Visconti *et al*., 1995).

The stimulation of capacitation by direct addition of H_2O_2 is associated with the stimulation of protein tyrosine phosphorylation (Aitken, 1997). The mechanism of H_2O_2-induced tyrosine phosphorylation may involve the inhibition of tyrosine phosphatase activity or the stimulation of tyrosine kinase activity (for review, see Aitken, 1997). The link between PKA and protein tyrosine phosphorylation is of interest since, among the human sperm protein substrates for tyrosine phosphorylation are pro-AKAP82 and AKAP82, members of the A-kinase anchor protein (AKAP) family (Carrera *et al*., 1996). AKAPs bind to the RII subunit of PKA, thereby recruiting the kinase to its physiological substrates, and indicating that additional interactions between PKA and tyrosine kinase signalling pathways are possible (Fig. 2).

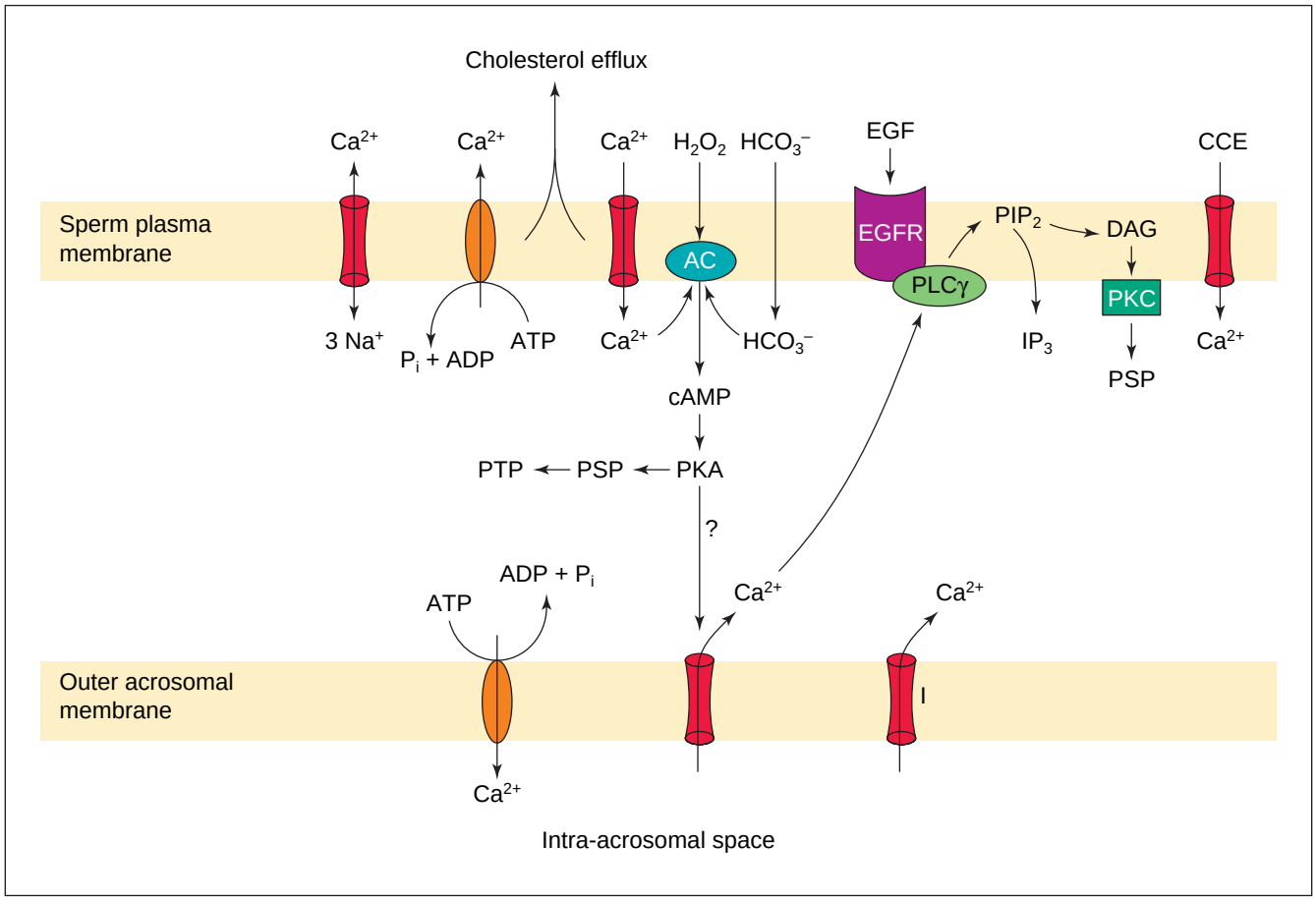

Fig. 2. Possible interactions among the activities invoked during the sperm capacitation process. In the female reproductive tract, the sperm cell is exposed to various components, among them epidermal growth factor (EGF), which can activate its receptor (EGFR). As a result, there is a gradual increase in phospholipase Cγ (PLCγ) binding to the plasma membrane during capacitation. Proteins in the female tract will cause cholesterol efflux from the sperm plasma membrane, leading to an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), HCO_3^- and H_2O_2, resulting in the activation of membrane-bound adenylyl cyclase (AC) to produce cAMP. The cAMP activates protein kinase A (PKA) to phosphorylate proteins on serine (PSP), leading to protein tyrosine phosphorylation (PTP). It is possible that PKA also activates the Ca^{2+} channel of the outer acrosomal membrane, resulting in increased $[Ca^{2+}]_i$ and the activation of EGFR-coupled PLC. Since PLC is gradually activated during capacitation, and $[Ca^{2+}]_i$ at the end of the capacitation is relatively low (160 ± 40 nmol l^{-1}), it is assumed that the IP_3-dependent Ca^{2+} channel (I) and the capacitative Ca^{2+} entry (CCE) are not fully activated at this point. These two channels are activated later after the binding of the spermatozoon to the zona pellucida of the egg.

The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, has been identified in the head of the bovine spermatozoon (Lax *et al.*, 1994). The EGF-induced acrosome reaction in capacitated bovine spermatozoa is almost completely blocked by tyrosine kinase inhibitors (Lax *et al.*, 1994; Table 2), indicating the involvement of protein tyrosine phosphorylation in the acrosome reaction (Fig. 2). The EGFR may also play a role during sperm capacitation since EGF stimulates the tyrosine phosphorylation of several proteins (Breitbart *et al.*, 1995) and activates phospholipase C (PLCγ), two steps that occur during sperm capacitation (Spungin *et al.*, 1995). In addition, activation of PLCγ or the acrosome reaction by the zona pellucida are also modulated by tyrosine phosphorylation (Leyton *et al.*, 1992; Tomes *et al.*, 1996). A tyrosine kinase that may be involved in the acrosome reaction is the 95 kDa protein that binds to zona pellucida in mouse spermatozoa (Leyton and Saling, 1989). A similar 95 kDa protein has been found in human spermatozoa, and the tyrosine phosphorylation of this protein is enhanced during capacitation (Burks *et al.*, 1995). Progesterone stimulates the tyrosine phosphorylation of a 94 kDa protein in human spermatozoa (Tesarik *et al.*, 1993) and this protein may also be a tyrosine kinase receptor. However, the nature of the 95 kDa protein and the sperm–zona-pellucida receptors are controversial issues (Bork, 1996; Saling *et al.*, 1996; Tsai and Silver, 1996). Nevertheless, there is general agreement that protein tyrosine phosphorylation increases during capacitation and the acrosome reaction and, hence, that it may participate in sperm activation.

Another candidate for tyrosine phosphorylation is the sperm GABA-like receptor–Cl^- channel involved in the Cl^- efflux mediated by progesterone during the acrosome reaction (Sabeur *et al.*, 1996). Since lavendastin A, a tyrosine kinase

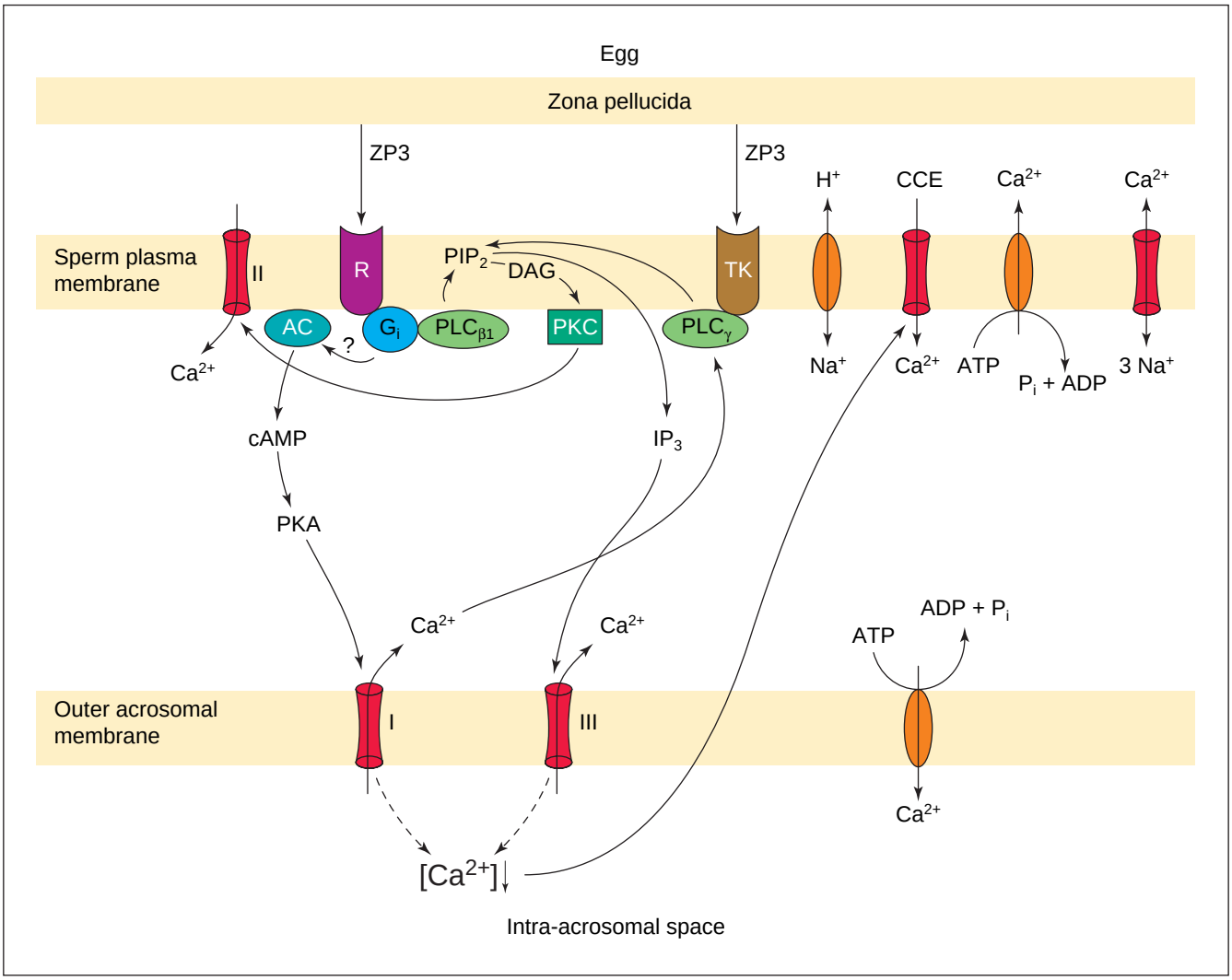

Fig. 3. Possible interactions among the activities invoked during the acrosome reaction. The glycoprotein ZP3 binds to at least two different receptors in the plasma membrane. One (R) is a G_i-coupled receptor that activates phospholipase Cβ1 (PLCβ1). The other is a tyrosine kinase (TK) receptor coupled to PLCγ. Binding to R would regulate adenylyl 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, increase 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) 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 $[Ca^{2+}]_i$. In addition, the PKA and IP_3-dependent Ca^{2+} channels (I and III) of the outer acrosomal membrane will cause acrosomal Ca^{2+} depletion, leading to the activation of a capacitative Ca^{2+} entry (CCE) mechanism in the plasma membrane. The G_i or TK can also activate an Na^+–H^+ exchanger in the plasma membrane, leading to the alkalization of the cytosol. The increases in $[Ca^{2+}]_i$ and pH_i will lead to membrane fusion and acrosomal exocytosis.

inhibitor, inhibits the progesterone-initiated human sperm acrosome reaction, it is possible that progesterone mediates the tyrosine phosphorylation of the sperm GABA-like receptor–Cl^- channel during sperm activation (Meizel and Turner, 1996).

The induction of the boar sperm acrosome reaction by the Ca^{2+} ionophore A23187 causes a significant decrease in the tyrosine phosphorylation of 93, 175 and 220–230 kDa proteins, indicating that dephosphorylation of tyrosine phosphate may be associated with Ca^{2+} influx during the acrosome reaction (Kalab *et al.*, 1998). Differences in the regulation of cAMP and protein tyrosine phosphorylation in mouse, human and boar spermatozoa indicate that the sensitivity to $[Ca^{2+}]_i$ and concentrations of cyclic nucleotide phosphodiesterase may correspond to species-specific regulatory strategies in mammalian spermatozoa (Kalab *et al.*, 1998).

Protein kinase C

Active PKC is present in human, bull and ram spermatozoa (Breitbart *et al.*, 1992). Immunocytochemical studies localize PKC

Table 2. Effect of several inducers and inhibitors on the bovine sperm acrosome reaction (AR)

Inducer or inhibitor	TPA (1.6 nmol l^{-1}) AR%	I%	EGF (1 ng ml^{-1}) AR%	I%	dbcAMP (0.1 mmol l^{-1}) AR%	I%
None	26	—	34	—	30	—
Staurosporin (1 nmol l^{-1})	2	92	3	91	31	0
Tyrphostin (1 µmol l^{-1})	13	50	3	91	27	8
Lavendastin (5 µmol l^{-1})	22	15	9	74	29	3

Ejaculated bovine sperm (10^8 ml^{-1}) were capacitated for 4 h, incubated for 10 min with the inhibitor, and then the inducers were added for an additional 20 min, and the number of acrosome reacted cells were counted.
Values represent the percentage of acrosome reacted cells (AR%) or the percentage of inhibition (I%). The AR% without any inducer was 8%.
dbcAMP: dibutyryl cAMP; EGF: epidermal growth factor; TPA: 12-*O*-tetradecanoyl phorbol-13-acetate.

mainly to the equatorial segment of human spermatozoa (Rotem *et al*., 1990) while, in bull spermatozoa, the immunolabelling is seen mainly in the post-acrosomal and upper region of the head. In ram spermatozoa, immunolabelling is found in the upper region of the acrosome (Breitbart *et al*., 1992). The localization of PKC in the acrosomal region of the sperm head indicates the possible involvement of PKC in the acrosome reaction. In support of this hypothesis, the PKC activator phorbol myristoyl acetate (PMA) stimulates the acrosomal reaction in capacitated bull and human spermatozoa in a PKC-dependent mechanism (Breitbart *et al*., 1992; Rotem *et al*., 1992) (Fig. 3). Several lines of evidence support the hypothesis that PKC is involved in the stimulation of acrosome reaction elicited by PMA and physiological ligands (Naor and Breitbart, 1997). Less potent tumour promoter phorbol esters, such as 4β-phorbol-12,13 didecanoate (4β-PDD), are less active in eliciting acrosomal exocytosis induction, whereas inactive analogues, such as 4α-phorbol-12,13 didecanoate (4α-PDD), have no effect on the sperm acrosome reaction. The response time for the induction of the acrosome reaction with PMA and the membrane-permeable diacylglycerol analogue, 1-oleoyl-2-acetyl glycerol (OAG), which is a specific PKC activator, is similar to that for PMA. Furthermore, the stimulatory effect of PMA on the acrosome reaction is abolished by PKC downregulation or by the use of PKC inhibitors (De Jonge *et al*., 1991b; Rotem *et al*., 1992).

Further support for the notion that PKC plays a role in sperm activation is derived from studies with physiological sperm ligands. The potential sperm ligands, ZP3 and progesterone, stimulate phosphoinositide turnover and the acrosome reaction in mammalian spermatozoa (Roldan *et al*., 1994; Liu and Baker, 1997). In addition, progesterone-stimulated protein phosphorylation is mediated by PKC (O'Toole *et al*., 1996a). These observations indicate that PKC is involved in the mechanism of the acrosome reaction under physiological conditions. The formation of diacylglycerol by ZP3 and progesterone is proposed to directly activate phospholipase A2 (Roldan *et al*., 1994), while it is suggested that sperm PKC is the site of diacylglycerol action and that phospholipase A2 activation occurs downstream of PKC activation.

Additional support for this hypothesis comes from the finding that the acrosome reaction or the production of prostaglandin $PGF_{2\alpha}$ induced by PGE_2 or melittin are both inhibited by phospholipase A_2 and PKC inhibitors (Breitbart *et al*., 1995). This inhibitory effect is blocked by the addition of exogenous arachidonic acid, supporting the hypothesis that PKC acts upstream of phospholipase A2 activation.

Separate mechanisms may be involved in the PMA- versus the Ca^{2+} ionophore-induced acrosome reaction in human spermatozoa (Rotem *et al*., 1992). The PMA-induced acrosome reaction is abolished by PKC inhibition or by depletion of endogenous PKC, but not by Ca^{2+} removal. In contrast, the ionomycin-induced acrosome reaction is blocked by Ca^{2+} removal, but not by PKC inhibition or depletion. Therefore, it appears that separate mechanisms for the Ca^{2+} increase or PKC activation are involved in the acrosome reaction induced by ionomycin or PMA. Potential sperm ligands, such as ZP3, may use both pathways during sperm activation. Furthermore, the two pathways may merge during ligand action (Fig. 3), since PKC is thought to activate a plasma membrane Ca^{2+} channel to generate a secondary increase in intracellular Ca^{2+} concentrations (Spungin and Breitbart, 1996). Moreover, cross-talk with cAMP signalling appears to occur, since PKA generates the first increase in Ca^{2+} concentrations by acting on an acrosomal Ca^{2+} store (Spungin and Breitbart, 1996; Fig. 3). The Ca^{2+} channel blocker, nifedipine, inhibits progesterone-induced PKC-mediated protein phosphorylation (O'Toole *et al*., 1996a) and diacylglycerol production (O'Toole *et al*., 1996b), indicating that PKC activation occurs downstream of Ca^{2+} entry. Direct measurement of Ca^{2+} influx into human spermatozoa indicates that the progesterone-stimulated $[Ca^{2+}]_i$ increase is independent of PKC, supporting the hypothesis that Ca^{2+} influx is upstream of the activation of PKC (Bonaccorsi *et al*., 1998). This finding appears to contradict the suggestion that PKC activates a plasma membrane Ca^{2+} channel (Spungin and Breitbart, 1996), indicating that the Ca^{2+} influx is downstream of PKC activation. PKC activation of the plasma membrane Ca^{2+} channel is detected in plasma membranes isolated from bovine spermatozoa (Spungin and Breitbart, 1996). In these cells, there is no effect of progesterone on the acrosome reaction or Ca^{2+} influx, indicating the lack of progesterone receptor in bovine spermatozoa. Thus, the mechanism for Ca^{2+} entry of bovine spermatozoa may differ from the progesterone-dependent Ca^{2+} entry mechanism described

in human spermatozoa. Alternatively, Ca^{2+} may act before and after PKC activation (Fig. 3). Cytoskeletal elements involved in the acrosome reaction may be phosphorylated by PKC, enabling the rearrangement of the cytoskeleton under the available Ca^{2+} concentrations (Spungin *et al*., 1995). Another role that has been suggested for PKC in mediating the acrosome reaction is the activation of phospholipase A_2 and the generation of arachidonic acid and its products, which have been implicated in the acrosome reaction mechanism (Roldan *et al*., 1994).

Preliminary studies on sperm PKC subspecies distribution have revealed the presence of PKCα and PKCβII in the equatorial segment of human spermatozoa (Rotem *et al*., 1992), while PKCα and PKCβII are localized to the plasma membranes of bovine spermatozoa (Lax *et al*., 1997). PKCα and PKCβII may participate in the mediation of the acrosomal exocytotic reaction since both PKC isoforms participate in exocytotic responses in pituitary and PC12 cells (Naor *et al*., 1989; Ben-Shlomo and Naor, 1991). The presence of PKCβI and PKC in the principal piece of the sperm tail raises the possibility that both isoforms are also involved in sperm flagellar motility (Rotem *et al*., 1992). The localization and compartmentalization of sperm PKC isoforms indicate that they may be involved in specific substrate phosphorylation in distinct sperm regions to regulate separate physiological functions.

In human spermatozoa, PKC is immunolocalized in patches along the acrosome, the equatorial segment and the post-acrosomal dense laminae (Kalina *et al*., 1995). Immunolabelling of the sperm flagellum is observed in patches along the tail. The sperm neck shows staining at the basal plate, the segmented columns and the centriole. Patches of labelling are found along the mid-piece and principal piece of the tail. In both regions, immunolabelling is distributed between the elements of the axoneme and the outer dense fibres, whereas the mitochondria in the midpiece and the fibrous sheath of the principal piece are devoid of labelling.

Colocalization of PKC with its substrates may also be achieved by transfer of PKC from one compartment of the cell to another. This possibility is supported by the observation that the treatment of bovine spermatozoa with PMA results in a rapid and extensive translocation of cytosolic PKCα and PKCβI to the plasma membrane (Lax *et al*., 1997). Thus, the translocation of PKC is confirmed by the observation that PMA evokes a decrease in cytosolic PKC activity that is accompanied by an increase in membrane activity (Lax *et al*., 1997). This translocation of PKC ensures the phosphorylation of relevant and specific substrates and may determine its physiological functions.

Human spermatozoa have a highly specialized cytoskeletal organization. The localization of spectrin in the acrosome, annexins, vimentin and fibronectin in the equatorial segment, myosin in the neck and actin and spectrin in the principal piece of the tail may be relevant to potential PKC isoforms that are also localized in these regions. The colocalization of PKC within these cytoskeletal elements provides the potential substrates for sperm PKC subspecies. Indeed, several cytoskeletal elements are known to be PKC substrates *in vitro* (Kiley *et al*., 1992). Depolymerization of sperm F-actin is involved in the acrosome reaction (Spungin *et al*., 1995). Since both membrane fusion (Spungin *et al*., 1995) and actin depolymerization (Spungin and Breitbart, 1996) require supramicromolar Ca^{2+}, it is thought that the actin filaments constitute the final barrier to membrane fusion during acrosomal exocytosis. PKC phosphorylation of selective substrates may reduce the Ca^{2+} concentrations required for the acrosome reaction.

Integration of signalling

A ligand-independent increase in cAMP and PKA activation is involved in protein tyrosine phosphorylation during sperm capacitation (Visconti *et al*., 1995). After binding to the egg zona pellucida, a ligand-dependent increase in cAMP and the activation of PKA stimulate 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 initial increase in Ca^{2+} concentrations may support phospholipase C activation. The breakdown of PIP_2 by sperm ligands, such as ZP3 and progesterone, may remove the inhibition of actin-severing proteins to enhance the fusibility of the membranes. The formation of IP_3 may further increase Ca^{2+} concentrations by mobilizing an acrosomal Ca^{2+} pool, leading to the activation of capacitative Ca^{2+} entry. The increase in diacylglycerol concentrations activates specific PKC isoforms to activate a sperm plasma membrane Ca^{2+} channel (Spungin and Breitbart, 1996). PKC-mediated protein phosphorylation and the Ca^{2+} increase may activate actin-severing proteins, leading to the dispersion of F-actin and the fusion of the plasma and outer acrosomal membranes, culminating in acrosomal exocytosis. The localization of PKC in distinct structures of the human spermatozoon and the functional studies described here indicate a role for PKC in sperm physiology.

The active collaboration of R. Rotem, M. Kalina, B. Spungin, Y. Lax and S. Rubinstein is greatly appreciated.

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Key references are indicated by asterisks.

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