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THE cAMP-DEPENDENT KINASE PATHWAY AND HUMAN SPERM ACROSOMAL EXOCYTOSIS

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1. ABSTRACT

The human sperm acrosome reaction (AR) appears to be analogous to various somatic cell exocytotic events. The AR can be induced *in vitro* by naturally occurring and synthetic compounds, such as, human periovulatory follicular fluid (hFF) and calcium ionophore A23187. The events that culminate in the AR appear to involve at least two second messenger pathways. One pathway involves the generation of the second messenger adenosine 3':5'-cyclic monophosphate (cAMP) by the amplifying enzyme adenylate cyclase and leading to the activation of cAMP-dependent kinase (PKA). The effect of PKA stimulators, such as, forskolin and cAMP analogues, on AR was tested and they were found to stimulate the AR. Inhibitors of specific components in the PKA pathway, *e.g.* adenosine analogues and PKA inhibitors, induced dose-dependent reductions in the AR. Furthermore, naturally occurring agonists, including, hFF and solubilized human zona pellucida (sZP), in combination with PKA inhibitors, led to a significantly lower AR. Collectively, these data provide strong support for the role of the PKA pathway in the AR.

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2. BACKGROUND

2.1. Capacitation

From all cells, only the human spermatozoa in seminal plasma have the potential to fertilize the ovum. It is not until they are removed from seminal plasma, either by passage through cervical mucus or *in vitro* washing techniques, that spermatozoa become competent to fertilize a fully developed human oocyte. It is thought that proteins present in seminal plasma stabilize the sperm plasma membrane. These proteins also may serve to occupy receptors integral to the exocytotic process until such a time when it is beneficial for them to be shed, *e.g.*, after sperm passage through the cervix. A number of different terms, such as, decapacitation factor and acrosome stabilizing factor, have been applied to these proteins. It is at the time when the aforementioned proteins have been removed that changes begin to occur in the spermatozoon that prepare it for fertilization. These processes, collectively termed capacitation, were first described by Chang and Austin (1,2). Since these initial reports, a large number of review articles have been published addressing the physicochemical aspects of capacitation (3-23). While it is acknowledged that capacitation is a requisite preparatory process for fertilization, no clear recognizable marker for capacitation can be identified. At present, the only available marker to indicate the completion of capacitation is the occurrence of the acrosome reaction (see below). However, several characteristics have been associated with capacitation, and they are: 1) an increase in membrane fluidity, perhaps

facilitated by the removal of seminal plasma proteins; 2) a decrease in the plasma membrane cholesterol to phospholipid ratio; 3) a decrease in net surface charge; 4) an increase in oxidative processes and production of cAMP; and 5) changes in swimming patterns of sperm. When sufficient time is allowed for capacitation to occur (the duration of this period is dependent upon the incubation conditions) sperm become susceptible to stimuli that will induce a process called the acrosome reaction.

2.2. Acrosome reaction

The acrosome reaction is an exocytotic process and is a necessary step for successful penetration of the oocyte vestments and fertilization. On the basis of a number of investigations, it has long been held that mammalian spermatozoa must undergo a capacitative process, otherwise the acrosome reaction would not take place. However, this idea has recently been challenged by several investigators who have demonstrated that the human sperm acrosome reaction can be biochemically stimulated in a non-capacitated spermatozoa (24,25). Potentially, this occurs by bypassing membrane-mediated events. The conclusion from these and other findings is that capacitation and the acrosome reaction are distinct and distinguishable processes.

Morphologically, the acrosome reaction is characterized by point fusions between the plasma membrane and outer acrosomal membrane. These fusions are followed by the formation of fenestrations and hybrid vesicles. The vesicles consist of both plasma and outer acrosomal membranes. The interior of the acrosome, termed the acrosomal matrix, becomes exposed and solubilized by processes that are not completely understood. Upon or coincident with solubilization, an acrosomal serine glycoproteinase is converted from the zymogen form of the enzyme, proacrosin, to its active form, acrosin. Acrosin is thought to be a key enzyme for facilitating sperm binding and penetration through the oocyte vestments (10,12-14,19-22,26-31).

2.3 Communication between sperm and egg

Surrounding the oocyte is an acellular glycoprotein matrix called the zona pellucida. The zona pellucida serves as a species specific barrier for fertilization. In addition, it is commonly held that a zona pellucida glycoprotein, termed ZP3, is primarily responsible for acrosome reaction induction (21,27,28). It is critical that the sperm membrane receptor or receptors for ZP3 be properly situated and primed to allow for the appropriate signaling and subsequent cascade of events that will culminate in the acrosome reaction, and lead to the localized activation and release of the acrosomal enzymes (19-22,27-31). A breakdown in this sequence of events can influence the outcome of fertilization. On the other hand, follicular and oviductal fluids, and cumulus oophorus can induce the acrosome reaction (30-35) in certain spermatozoa that are not yet temporally or spatially competent to fertilize an ovum. Furthermore, dysfunction in the intracellular signaling processes of the acrosome reaction (22,36-38) is likely to lead to reduced fertilization potential of the spermatozoon.

2.4 Model for intracellular signal-induced acrosomal exocytosis

The acrosome reaction is analogous to exocytotic processes that occur in various somatic cells, such as, nerve terminals and mast cells. The exocytotic process in human spermatozoa appears to involve the following sequence of events (21,22,26,31,32,36-38,42-53). The interaction of an extracellular ligand, *e.g.*, ZP3, with a sperm membrane-bound receptor causes a conformational change in a receptor-linked guanine nucleotide binding protein, G-protein. The G-protein influences one or more targets, including: ion channels, other G-proteins or membrane-associated amplifying enzymes (39,40). If the G-protein interacts with an amplifying enzyme, *e.g.*, adenylyl cyclase, then the result is the hydrolysis of a precursor molecule, *e.g.*, adenosine 5'-triphosphate (ATP), into a second messenger molecule, *e.g.*, adenosine 3':5'-cyclic monophosphate (cyclic AMP). The second messenger activates a protein kinase, *e.g.*, cAMP-dependent kinase, and thus phosphorylation of a protein integral to the exocytotic process (41). It is puzzling that no clear link between G-protein and target amplifying enzyme, second messenger or protein kinase has yet been demonstrated. Alternatively, several second messenger pathways have been shown to participate in the acrosome reaction (21,22,26,31,32,36-38,42-53).

This review will describe results from several reports in which the addition of cAMP analogues (dibutyryl cyclic AMP, 8-bromo cAMP), a stimulator of cAMP production (forskolin) or phosphodiesterase inhibitors (isobutylmethy lxanthine, papaverine, SQ 20009) to capacitated human spermatozoa culminates in the acrosome reaction. In addition, the use of inhibitors (adenosine, 2'-O-methyladenosine, 2',3'-dideoxyadenosine, KT5720, H-8, Walsh cAMP-dependent protein kinase inhibitor) that target specific pivotal enzymes in the cAMP-dependent kinase (PKA) pathway prevent induction of the AR when upstream components of the pathway are stimulated. These findings give credence to the possible role of the cAMP-dependent kinase signal transduction pathway in human sperm acrosomal exocytosis.

3. EXPERIMENTAL EVIDENCE FOR PKA INVOLVEMENT IN THE ACROSOME REACTION

3.1 Assay conditions

A procedure used for testing the effect(s) of stimulators and inhibitors on the acrosome reaction is the synchronous acrosome reaction assay (54). In this assay, spermatozoa are incubated for 3 hours at 37ºC in 5% CO_2 atmosphere to induce capacitation. An acrosome reaction stimulator is then added to one of two tubes and the incubation is continued for an additional 15 minutes. An aliquot of capacitation medium containing spermatozoa is removed from each tube and assessed for the percentage of motile cells ("motility") before stopping the reaction. Inhibitors of the acrosome reaction are added at the end of the incubation period, *i.e.*, after spermatozoa had become capacitated, 5 minutes prior to the addition of activator and the assay is continued as described above.

3.2 cAMP and calcium

The first step in the acrosome reaction, after the spermatozoa are fully capacitated, is generally considered to be an influx of exogenous calcium ions across the sperm membranes. The addition of exogenous calcium alone to capacitated human spermatozoa does not stimulate the acrosome reaction (37). However, the use of a calcium transporting agent, such as, calcium ionophore A23187, to facilitate calcium entry results in a significant stimulation of the acrosome reaction (37, 54). However, calcium ionophores, such as A23187, have been shown to cause the liberation of calcium from internal stores, which contributes to the exocytotic process (*e.g.*, 57). An argument against this concept is that A23187 is unable to induce an acrosome reaction when calcium is not included in the capacitation medium (37), suggesting that intracellular storage sites for calcium that can effect an acrosome reaction may not exist in human spermatozoa. By contrast, dbcAMP does induce the AR in the nominal absence of calcium, indicating that dbcAMP is able to bypass the calcium transporting requirement (37). These findings can be interpreted to mean that cAMP exerts its effect after the influx of calcium during the cascade of events which lead to acrosomal exocytosis. This has been shown to be the case for some somatic cell processes (*e.g.*, 41). Therefore it is possible that in human spermatozoa an influx of calcium either directly or indirectly activates adenylate cyclase, resulting in an increase in cAMP levels.

Addition of xanthine and non-xanthine phosphodiesterase inhibitors to capacitated spermatozoa stimulates the AR to the same extent as the cAMP analogues (37). However, methylxanthines have also been shown to alter calcium homeostasis and transport. Yet based on the diversity of the phosphodiesterse inhibitors used, and their varying specificity's, it can be reasoned that the stimulatory effect on the AR was due to an increase in cAMP levels rather than a change in Ca^{2+} transport. Furthermore, in contrast to some other cells, cAMP does not function by increasing the Ca^{2+} transport across the cell membrane (58).

3.3 Adenylate cyclase

Forskolin is a diterpine isolated from the Indian plant *Coleum forskoli*. In isolated cell membranes and intact cells from a variety of mammalian tissues, forskolin has been shown to activate adenylate cyclase (*e.g.*, 59). The same activating response would appear to be the case when capacitated spermatozoa are treated with forskolin. Forskolin stimulates the acrosome reaction of capacitated human spermatozoa, and to the same extent as the cAMP analogues (37). The ability of an adenylate cyclase activator to stimulate the AR strongly suggests that adenylate cyclase has a role in the AR of human spermatozoa.

Adenosine interacts with adenylate cyclase at two locations. One position, called the "R-site," has properties associated with extracellular hormone receptor-mediated responses of the membrane adenylate cyclase. The second position, which is not a cell surface receptor, is called the "P-site" and mediates the inhibition of adenylate cyclase (60). The addition of adenosine and two adenosine analogues, 2'-O-methyladenosine and 2',3'-dideoxyadenosine, to spermatozoa prevented stimulation of the acrosome reaction by forskolin and independent of whether they were added at the onset of incubation or after incubation to induce capacitation (37). The latter two inhibitors have been shown to act as agonists of the P-site (60). Since the two adenosine analogues inhibited the forskolin-induced AR, it is possible that the sperm cyclase is regulated via a P-site. Further evidence for the involvement of the adenylate cyclase/cAMP pathway in the human sperm AR comes from the ability of dbcAMP to overcome adenosine inhibition and stimulate the reaction. These results offer compelling support for the involvement of adenylate cyclase in the acrosome reaction.

3.4 cAMP-dependent kinase

Inhibitors of cAMP-dependent kinase (PKA) typically bind to the catalytic subunit causing the displacement of the regulatory subunit thereby inhibiting the phosphorylating activity of the kinase. Inhibitors with good specificity for their target enzyme, *i.e.*, kinases, have recently been shown to prevent stimulation of the AR by compounds that act at that same target or a target upstream from the site

of inhibition (37). KT5720, a competitive and reversible inhibitor of PKA, completely prevented stimulation of the AR by forskolin and by dbcAMP when used at a maximum test concentration (100nM). However, two lower concentrations of KT5720 (25nM and 50nM) caused a 58% inhibition of the forskolin-induced AR and a 50% and 67% inhibition of the dbcAMP-induced AR (37). The dose-dependent decreases in the AR achieved using KT5720 would appear to be reflective of an overall decrease in PKA activity. Furthermore, the concentrations used that resulted in the *in vivo* inhibition of PKA, *i.e.*, referring to inhibition of the enzyme in the viable cell and not of the extracted enzyme, are commensurate with the reported inhibition constant for this compound (Ki=0.056 mM) as tested on somatic cell PKA *in vitro* (62).

3.5 Solubilized zona pellucida

The AR is initiated during the penetration of the spermatozoon through the follicle cell layer of the oocyte, either just prior to or after contact with the zona pellucida. At present, although the oocyte stimulus or stimuli of the AR remain to be definitively identified, it is likely that they include one or more of the zona glycoproteins.

It has been shown that the human sperm AR can be induced by solubilized human zonae (sZP)(46). Further, when several PKA inhibitors were tested to determine if the signal transduction pathway stimulated in the spermatozoon by sZP might involve the cAMP-dependent kinase pathway, a significant reduction in the AR was detected. For example, the addition of KT5720 to medium containing sperm, and just prior to addition of sZP, caused a reduction in the AR but not to the same level as the control. However, when a combination of inhibitors of different kinases was tested, the sZP-induced AR was reduced to levels approaching that of the control. These results suggest that human sZP induces the AR via stimulation of kinases from three different signaling pathways, and one of which appears to be the cAMP-dependent kinase pathway.

3.6 Follicular fluid

Previous data have shown that human follicular fluid stimulates an acrosome reaction in capacitated human spermatozoa (32-35). When an inhibitor of protein kinase A (KT5720) was added to spermatozoa at the end of the capacitation period and 5 min prior to the addition of inducer complete inhibition of the AR occurred (32). These data suggest that periovulatory hFF stimulates the human sperm AR and by activating the cAMP-dependent kinase pathway. Finally, it is clear that additional work is required to delineate the precise sequence in which the PKA pathway becomes involved in the exocytotic process following exposure to either hFF or ZP3.

4. CONCLUSION

In conclusion, the present review provided evidence for the involvement of the cAMP-dependent kinase pathway in the human sperm acrosome reaction. It is likely that the mediation of one or more external signals by the sperm plasma membrane results in the activation of this pathway after or simultaneous with the entry of calcium. Concurrent with or following calcium influx, adenylate cyclase becomes active, resulting in increased cAMP levels, activation of cAMP-dependent kinase, and protein phosphorylation. It remains to be established which proteins become phosphorylated and how they function in the AR. Further, the activation of the cAMP-dependent kinase pathway by biological effectors of the AR, such as, sZP3 and hFF, requires clarification.

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