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PKC IN EGGS AND EMBRYOS

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1. ABSTRACT

After a spermatozoon enters the egg, rapid processes such as the cortical granules exocytosis (CGE) occur. Other, later processes are observed as well, and they include resumption of the second meiotic division, second polar body (PBII) extrusion and rearrangement of the DNA inside the pronuclei (PN). The above mentioned biochemical processes involve signal transduction pathways that are well known in other cell systems, and require mediation of second messengers like Ca^{2+} and diacylglycerol (DAG) which are protein kinase C (PKC) activators. The present review, based upon recently published studies, raises the possibility of PKC involvement in fertilization and in early developmental stages of the mammalian embryo.

2. INTRODUCTION

2.1. Egg maturation and activation

Meiosis in the mammalian egg demonstrates two meiotic onsets. One arrest is at the prophase of the first meiotic division, when the genetic material organizes in a specific structure known as the germinal vesicle (GV). The second arrest is at the metaphase of the second meiotic division (MII). The resumption of the first meiotic division occurs in the mature female after exposure to a preovulatory LH surge. This process is referred to as maturation and it takes place only in a small number of eggs each cycle. The meiotic resumption is characterized by germinal vesicle break down (GVBD), completion of the first meiotic division, extrusion of the first polar body (PBI) and initiation of the second meiotic division up to the MII stage. The egg is ovulated at the MII stage, and awaits the fertilizing spermatozoon at the ampullary region of the oviduct (1). The fertilizing spermatozoon penetrates through the cumulus cells and the zona pellucida (ZP) and its membrane fuses with the oolema driving the egg to activation in a sequence of intracellular events. These events are preceded by cleavage of phosphatidylinositol (PIP_2), yielding two products: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (2). IP_3 production leads to Ca^{2+} release from intracellular stores and the DAG could activate the enzyme protein kinase C (PKC) (3, 4).

Activation events within the egg are classified as early and late events.

2.1.1 Early events

(a) Cortical granule exocytosis (CGE) – the content of the cortical granules (CG) is expelled and causes biochemical changes of the ZP that prevent penetration of additional spermatozoa (5,6,7).

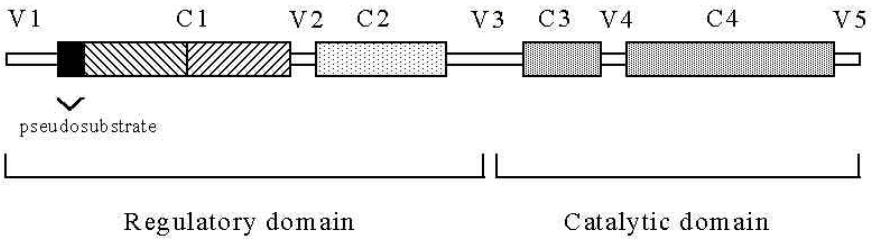

Figure 1. PKC structure (Based on Newton 1997).

(b) Resumption and completion of the second meiotic division culminating in the extrusion of the second polar body (PBII) (8).

2.1.2. Late events

Pronuclei (PN) formation, DNA synthesis, activation of maternal mRNA for protein synthesis (9).

The first observable cellular event, after the gamete fusion, is a rapid increase in intracellular calcium concentration ($(Ca^{2+})_i$) followed by repetitive $(Ca^{2+})_i$ oscillations (10,11,12). The initial $(Ca^{2+})_i$ rise is a result of $(Ca^{2+})_i$ release from intracellular stores and is sufficient for inducing egg activation (13,14,15). The induction of $(Ca^{2+})_i$ rise by external activators, such as Ca^{2+} ionophore or electric stimulation, causes parthenogenetic egg activation presenting both CR and resumption of meiosis (11,16,17). The addition of a Ca^{2+} chelator that prevents the $(Ca^{2+})_i$ rise, inhibits both CR and resumption of meiosis. It was suggested that the initial $(Ca^{2+})_i$ is released from intracellular stores at the endoplasmatic reticulum (ER) that have receptors for IP_3 (16,18,19). Specific antibodies against IP_3 inhibited both events of egg activation (20). In addition, a single injection of IP_3 causes CR and ZP changes (21,22), but does not cause resumption of meiosis and of cell cycle (23,24). Further study that investigated this point demonstrate that low Ca^{2+} concentrations allow CG exocytosis, while higher levels are required for cell cycle resumption (25). Deducible conclusions based on these results are that CR and resumption of meiosis are dependent on different levels of $(Ca^{2+})_i$, and that sperm penetration activates a number of vital pathways that lead to the completion of egg activation.

It is yet not clear how the spermatozoon initiates a $(Ca^{2+})_i$ rise in the egg. Two main hypotheses attempt to explain the process:

a) The receptor hypothesis - Binding of the spermatozoon by a ligand to a specific receptor on the oolema, transduces a signal that mediates a cascade of cellular events. It was suggested that the spermatozoon could bind either to a G-protein-coupled receptor or to a tyrosine kinase receptor. Although such receptors, which are present on the oolema, could mediate artificial activation of the eggs when triggerd by activators, various studies failed to support their involvement during fertilization (9,26,27).

b) The sperm factor hypothesis - Fusion between the spermatozoon and the egg membrane allows the delivery of a sperm component into the ooplasm that is capable of inducing the early events of egg activation (2). Injection of sperm extract into mammalian eggs, induced the initial Ca^{2+} rise, Ca^{2+} oscillations and a full activation of the egg followed by development to the blastocyst stage (28).

2.2. PKC – structure and function

2.2.1. Enzyme structure

PKC is active in a vast array of cellular responses such as: exocytosis, receptor regulation, gene expression, muscle contraction and relaxation, activation of ionic channels and cell differentiation and transformation. PKC posses enhancing and inhibiting activities, thus making it possible for the enzyme to enroll in positive activation and/or in negative feedback (3,29,30). PKC is represented by a family of serine and/or threonine kinases sharing a similar basic structure - one subunit of 77-83 kDa. Differences were described among the isoenzymes in regards to the kinetics of their activity, their expression in the various tissues and their target proteins (31,32).

The isoenzymes of this family are classified into three groups according to their structure and demands for activation (33).

Group 1: The conventional PKC (cPKC) that are Ca^{2+} and DAG dependent – alpha, beta I, beta II and gamma.

Group 2: The novel PKC (nPKC) that are Ca^{2+} independent and DAG dependent – delta, epsilon and theta.

Group 3: The atypical PKC (aPKC) that are Ca^{2+} and DAG independent –lambda, zeta, mu and iota.

All the various isoenzymes have a significant degree of homology containing conserved sites (C_1 – C_4) that comprise the functional sites and variable sites (V_1 – V_5). The variable sites are situated among the conserved site and their function is yet unclear (34,35) (Figure 1).

The protein sequence consists of a regulatory domain and a catalytic domain (Figure 1).

2.2.1.1. The regulatory domain

The C_1 site is present in all the isoenzymes except PKC zeta. This site is the binding domain of DAG and other activators like phorbol esters (36). An additional binding domain in this site binds phosphatidyleserine that acts as a cofactor in activating PKC.

The C_2 site characterizes the isoenzymes belonging to the cPKC group, the Ca^{2+} dependent isoenzymes, and it is the most probable site to contain a Ca^{2+} binding domain (37). The amino acid sequence of the V_1 site among the different isoenzymes is not conserved, thus leading to the assumption that this site is responsible for the selectivity in binding the protein substrate. Each one of the PKC isoenzymes has the ability to bind a limited number of protein substrates (37). The amino acid sequence 13-30 that is situated at the C_1 site is the pseudosubstrate domain, which is the enzyme's self-controlling (inhibition) domain. The inhibition is induced by interaction between the pseudosubstrate domain and the protein substrate-binding locus, which is situated at C_4 of the enzyme's catalytic domain. During enzyme activation, the pseudosubstrate domain detaches from the catalytic domain and enables the binding and phosphorylation of the appropriate substrate (38).

2.2.1.2. The catalytic domain

The domain is located at the carboxyterminal region of the enzyme. The C_3 site contains an ATP binding locus, whereas within the C_4 site there are two binding loci – one for the protein substrates and the other is for ATP (37).

2.2.2. Enzyme activation

The inactive PKC is dispersed in the cytosol. In many cell types, PKC activation is initiated after binding of a ligand to a receptor on the cell membrane. The ligand-receptor interaction is through a G-protein, which in turn activates PLC that hydrolyzes PIP_2 to IP_3 and DAG. IP_3 serves as a second messenger and triggers a $(Ca^{2+})_i$ rise. The binding of PKC to Ca^{2+} causes a conformational change of the enzyme, exposure of the hydrophobic domain and the migration of the enzyme towards the membrane. At the membrane PKC binds to DAG, undergoes an additional conformational change, followed by the release of the pseudosubstrate from the catalytic domain. The catalytic domain is then able to bind a protein substrate and to phosphorylate it on serine and/or threonine residues.

PKC contains three phosphate-binding regions, each of them has two autophosphorylation domains. The autophosphorylation enhances the enzyme's affinity to phorbol esters and to Ca^{2+} ions (39). Apparently, the enzyme's autophosphorylation enables its binding to the cell membrane and hence to its down regulation (40,41). The enzyme's autophosphorylation requires a lower phosphatidylserine concentration than that required for the substrate phosphorylation. The phosphatidylserine concentration determines the uniqueness of the bond between the enzyme and the substrate. During the binding to the phosphatidylserine the enzyme is either present in the cytoplasm or bound reversibly to the cell membrane in a Ca^{2+} dependent manner.

Physiological activation of PKC is made possible in two ways:

1. The cytoplasmatic PKC is a target for activation by unsaturated fatty acids.

2. The membranous PKC is activated by DAG. Synergism between unsaturated fatty acids and DAG causes activation of several types of PKC in the presence of a basal $(Ca^{2+})_i$ concentration. PKC activation may be biochemically dependent on Ca^{2+} but at the same time physiologically Ca^{2+} independent, i.e. does not require a rise in $(Ca^{2+})_i$ (3,42,43).

A group of lipid component receptors in the cell membrane and in the cytoskeleton and metabolic components, can induce, enhance and lengthen PKC activity (44). These receptors are referred to as receptors for activated C-kinase (RACKs). Binding of PKC to RACK takes place only after the PKC activation. If the enzyme is not activated, the binding domains to RACKs are blocked by a sequence called pseudo-RACK (45). PKC activation leads to the disassembly of the intra-molecular interaction and enables the exposure of the PKC's RACK binding domain and the catalytic domain.

Phorbol esters, such as tetradecanoyl phorbol acetate (TPA), activate PKC in a manner similar to DAG, probably by binding to the same domain at the enzyme (37,44).

2.3. PKC isoenzymes in eggs and embryos

Several studies presented the expression of PKC isoenzymes in egg and early embryos. PKC alpha, beta I, beta II, gamma, delta and zeta were identified in Xenopus eggs (46). Eight PKC isoenzymes: cPKC alpha, beta and gamma; nPKC delta, epsilon and mu, aPKC -zeta and lambda were detected in rat eggs (47). cPKC alpha and gamma; nPKC delta and mu, aPKC -zeta and lambda were detected in unfertilized MII mouse eggs (48,49) and in embryos from the 2-cell to the 8-cell stage. Almost all the above mentioned isoenzymes (except cPKC alpha) were detected in the blastocyst as well. cPKC beta could not be detected neither in unfertilized eggs nor in early embryos up to the 8-cell stage (49). The different PKC isoenzymes exhibit different patterns of localization at the same developmental stage and throughout the early embryogenesis (49). In general, at the early developmental stages, i.e. –MII up to the 2-cell stage, most of the PKC isoenzymes are dispersed homogeneously throughout the cytoplasm and in the nucleus. At later stage, i.e. form 4-cell to blastocyst, most of them are located mainly at the cell membrane and less at the cytoplasm and nuclei of the blastomers (49).

2.4. PKC and egg activation

2.4.1. Activation of PKC during egg activation

Activation of PKC can be measured by biochemical assays or by immunohistochemistry. Translocation of PKC isoenzymes from the cytosol to the plasma membrane serves as a marker for activation. Immunohistochemistry of MII arrested rat eggs demonstrated expression of the three cPKC izoensymes cPKC alpha, beta I, beta II. Upon activation by TPA, the three cPKC izosymes were translocated to the plasma membrane within several minutes (47). In another study performed in the mouse eggs fertilized *in vivo*, PKC translocation to the egg's membrane could be demonstrated

only at a later stage, upon PBII formation (about 40 min post sperm penetration) (50). In a recent work in mouse, the authors showed the translocation of PKC alpha and beta I, 10-60 minutes after *in vitro* fertilization but they were unable to demonstrate translocation of cPKC beta II. In addition, they demonstrated the translocation of PKC alpha but not of cPKC beta I or beta II after TPA treatment of GV eggs This activation was associated with the inhibition of GVBD (51).

2.4.1.1. $(Ca^{2+})_i$ rise

Addition of an extremely high concentration of TPA to mammalian eggs (mouse) , induces Ca^{2+} oscillations which are delayed by 10-30 minutes as compared to sperm induced Ca^{2+} oscillations (52,53,54). The high TPA dose required and the delay in the oscillation timing, imply that the signal at fertilization leading to Ca^{2+} oscillations is probably not activated by PKC (55).

2.4.1.2. Resumption of meiosis

The transition from metaphase II (MII) to anaphase II is initiated by Ca2+ elevation, and is PKC independent (56). The morphological events associated with the meiotic resumption include extrusion of PBII and formation of the pronuclei. TPA could induce PBII extrusion in hamster and mouse egg but the PB was quickly reabsorbed (57).

2.4.1.3. CGE

Activation of PKC by TPA induces CR, facilitates ZP modification and hence induces the block to polyspermy (22,58,59). However, when interfering with PKC pathways to CR during fertilization, PKC inhibitors failed to block fertilization induced CG release (60). These findings do not necessarily rule out the possibility of PKC involvement in exocytosis as either a very small amount of PKC is necessary for CR or that other active pathways can compensate for PKC inhibition.

These results raise a contradiction as to the possible role of PKC in mammalian egg activation. It is yet to be elucidated whether Ca^{2+} and PKC trigger separate pathways or whether they synergies in order to accomplish complete egg activation, including a maximal CR.

2.4.2. Activation of the egg by activating PKC

PKC is a key enzyme in the chain of cellular events and is active in many different cellular responses such as exocytosis or the signal transduction processes that require the involvement of serine/threonine kinase activation. It was reported that in different systems both signal pathways - Ca^{2+} elevation and PKC activation - are essential and often act synergistically to elicit full cellular responses (29). The universality of these findings indicates the possibility of PKC involvement in signal transduction pathways that take place during fertilization. Moreover, production of IP_3 and DAG, Ca^{2+} elevation and the exocytotic process that takes place as a result of sperm entry, further support this possibility.

In Xenopus eggs, activation of PKC induces CR without the resumption of meiosis. In addition, inhibition of PKC activation prevented CR even after activation by phorbol ester or ionophore (59). Unlike the Xenopus, in the sea urchin, the CR appears to be PKC independent (61).

2.5. The interaction between PKC and the cytoskeleton

Activation of the mammalian egg leads to reorganization of the cytoskeletal microtubles and actin filaments. Actin filaments in MII eggs are localized at the cortex, mainly above the meiotic spindle. Following egg activation, actin filaments depolymerize, the egg enters interphase, the cytoskeleton fibers migrate and assemble around the PN (mouse – 62,63). In the zebrafish it was demonstrated that egg activation leads to elevated amounts of F-actin and myosin-II and to their reorganization, few seconds prior to CGE (64).

The cytoskeleton proteins are functional targets for phophorylation by cAMP, Ca^{2+}-Calmodulin, Ca^{2+}–phosphatidylserine-DAG-dependent protein-kinases (65). Proteins like vinculin, talin, caldesmon and profilin, intermediate filaments like desmin and proteins accompanying microtubles like MAP2, are all phosphorylated by PKC (66,67,68,69). The use of PKC antibody in immunocytochemistry gives a signal similar to the one given by the use of vinculin antibody. In addition, the change in PKC localization after an exposure to phorbol esters simulates the change in the localization of vinculin and talin (70). PKC activation by TPA and DAG causes reorganization of actin in human neutrophils (71). PKC involvement in cytoskeleton reorganization may take place directly by phosphorylation of the actin fibers, or indirectly via phosphorylation of a PKC substrate that influence actin (72).

PKC activation by phorbol esters or by hormonal stimulus that causes hydrolysis of the PIP_2 in other cell systems, triggers activation and rescattering of PKC alpha and PKC beta, and reorganization of actin in the cytoskeleton. This reorganization is typical to exocytotic processes. It implies that PKC could be involved in the reorganization of the egg's cytoskeleton leading to CGE, in response to the sperm signal.

3. DISCUSSION

PKC could be involved in a number of processes during mammalian egg activation. These processes include CGE (22,58), structural changes that take place mainly in the spindle fibers and in the intermediate filament network (73) and the PBII formation (73,74). Still, its involvement in other developmental directions during egg activation and early embryonic development is not yet clear.

An important primary step towards revealing the role of PKC in the above mention process was when the expression of various PKC family members was demonstrated in the egg (47,48,49). A couple of studies showed the ability of several PKC isoenzymes to be activated during egg parthenogenetic activation or fertilization (47,50,51).

Fertilization in mammals is a long and a fairly complicated procedure, which includes a large number of

biochemical processes that demand the activation of various kinases at different times.

The presence of PKC representatives from all three groups in eggs and in early embryos, and their ability to be activated under certain biochemical requirements, imply that different isoenzymes could be involved in different events during fertilization and early development. However, it is not yet clear which PKC isoenzymes are involved at which process.

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