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Role of the phosphoinositide signal transduction pathway in the endometrium

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ABSTRACT

The regulation of calcium concentration triggers physiological events in all cell types. Unregulated elevation in calcium concentrations is often cytotoxic. In fact, uncontrolled calcium levels alter proteins' function, apoptosis regulation, as well as proliferation, secretion and contraction. Calcium levels are tightly regulated. A great interest was paid to signal transduction pathways for their role in mammalian reproduction. The role of phosphoinositide (PI) signal transduction pathway and related phosphoinositide-specific phospholipase C (PI-PLC) enzymes in the regulation of calcium levels was actively studied and characterized. However, the role of PI signaling and PI-PLC enzymes in the endometrium is far to be completely highlighted. In the present review the role of PI, the expression of selected PI-PLC enzymes and the crosstalk with further signaling systems in the endometrium will be discussed.

1. Introduction

The regulation of calcium concentration triggers physiological events in all cell types[1]. Unregulated elevation in calcium concentrations is often cytotoxic[2]. In fact, uncontrolled calcium levels alter proteins' function, apoptosis regulation, as well as proliferation, secretion and contraction. Calcium levels are tightly regulated by signal transduction pathways, including the phosphoinositide (PI) system [1,3].

A great interest was paid to PIs for their role in mammalian reproduction. In myometrium, the role of PI signal transduction pathway and related Phosphoinositide-specific Phospholipase C (PI-PLC) enzymes in the regulation of calcium levels was actively studied and characterized. Downstream products of PI-PLC activity are known to play a role in the contraction of uterine smooth muscle cells following the activation of muscarinic and oxytocin receptors in the guinea pig myometrium[4,5]. PI-PLC ζ enzyme is exclusively present in the spermatic cells. PI-PLC δ 4 is required for the zona pellucida-induced acrosome reaction[6]. In mice, the mutation of the gene which codifies for Pi-plc β 1 reduces the acrosome reaction rate, the fertilization rate and the embryo developmental rate[7].

Less in known about the role of PI signaling and PI-PLC enzymes in the endometrium. Reports from the '80s indicated that the PI signal transduction pathway plays a role also in the endocrine regulation of endometrium. In our recent report, we suggested that PI-PLC enzymes might be involved in endometriosis. In the present review the role of PI, the expression of selected PI-PLC enzymes and the crosstalk with further signaling systems in the endometrium will be discussed.

2. PI signal transduction pathway

PIs are minority acidic phospholipids present in the membranes of eukaryotic cells[8]. The principal role of PIs is instructional. Each cell membrane compartment uses a characteristic PI's pattern, which attracts a specific panel of proteins. PIs regulate the activities of integral membrane proteins. Many ion channels of the plasma membrane are regulated by the levels of phosphatidylinositol 4,5-bisphosphate (PIP2), which is the principal substrate of receptor-stimulated PI-PLC[9]. Over 150 different molecules can activate PI-PLC. Once activated, the PI-PLC cleaves the polar head group of PIP2, a structural component of the plasma membrane[9-11], generating inositol trisphosphate (IP3) and diacylglycerol (DAG), both crucial molecules in

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signal transduction[12]. IP3, a small water-soluble molecule, diffuses rapidly to the cytoplasm, where it releases calcium from the endoplasmic reticulum. DAG remains bound to the membrane, exerting two potential signalling roles[13]. First, it can be further cleaved to release arachidonic acid, which either acts as a messenger or is used in the synthesis of eicosanoids. Therefore, DAG is probably involved in the inflammation cascade. Moreover, recent evidences suggested that PI-PLC might be involved in the inflammation cascade[14]. Second, DAG activates protein kinase C (PKC). The calcium levels enhancement induced by IP3 moves PKC to translocate from the cytoplasm to the cytoplasmic face of the plasma membrane[15]. The translocation activates PKC, which phosphorylate specific serine or threonine residues on further target proteins[16].

3. PI-PLC family

In eukaryotes, the PI-PLC family comprises a related group of multi-domain proteins[17]. Thirteen mammalian enzymes were identified, divided into six sub-families on the basis of structure and activation mechanisms: β (1-4), γ (1, 2), δ (1, 3, 4), ε (1), ζ (1), and η (2)[17].

PI-PLC subfamilies share a conserved core architecture containing an *N*-terminal pleckstrin (PH), followed by a series of elongation factor hand motifs (EF), a complex catalytic domain (X-Y) and a *C*-terminal PKC conserved region 2 domain (C2)[18-20]. In the catalytic domain, the *N*-terminal X region, of 65 aminoacids, binds calcium. The *C*-terminal Y region, of 115 aminoacids, binds primarily the substrate.

Beside this common core, further domains, peculiar to each sub-family, contribute to specific regulatory mechanisms. Isoforms within sub-families share sequence similarity, specific domains, molecular effectors and targets, confirming the versatility of the interactions at the cellular membrane and its proximity.

4. PI-PLC regulation

The PI-PLC family of enzymes covers a broad spectrum of interactions that contribute to membrane recruitment, including binding to PI, interaction with small GTPases from Ras and Rho families or heterotrimeric GTP-binding protein (G protein) subunits, recognition of specific sites in receptor and non-receptor tyrosine kinases and interaction with lipid components of the cell membranes. Two major mechanisms were described in receptor-promoted regulation of PI-PLC enzymes[18-20].

G proteins: activating molecules, such as hormones and neurotransmitters stimulate G-protein-coupled receptors (GPCRs) to activate ubiquitously expressed members of G protein family. The G protein subunits are classified into subfamilies that couple to different effectors[21]. Three subunits (α, β, γ) comprise the G proteins. In the inactive state, the Gα subunit binds GDP. Receptor activation by ligand stimulates GDP-GTP exchange on the Gα subunit, and the activated GTP-bound Gα subunit dissociates from the Gβγ complex. Both the Gα and Gβγ subunits can influence the activity of effector molecules. In instance, PI-PLC β isozymes are directly activated by Gα-subunits of the Gq subfamily. G proteins mediate the intracellular signalling by coupling to GPCRs[22,23]. The tyrosine kinase-activated mitogen-activated protein kinase (MAPK) pathway can be activated by Gβγ acting at the Shc protein, by Gαq-stimulated PKC acting at Raf, and by a PKC-independent pathway[24]. GTPase activity in the GGTPase activity in the subunit promotes re-association of GDP-bound GGTPase activity in the subunit with Gβγ. The specificity of response to a given ligand in each cell type depends on the expression and coupling of specific receptors, G proteins, and effectors. In addition to direct stimulation by G proteins of effectors that initiate signalling pathways, there can be cross-talk among downstream effectors of these pathways[25]. Protein kinase A (PKA), activated by Gαs stimulation of adenylyl cyclase, can inhibit both PI-PLC activation and the MAPK pathway[26,27]. Also ion channels can be regulated by phosphorylation catalysed by PKA, PKC and other effectors of the G protein signalling pathway. GTPases: the Ras super-family of small GTPases comprises more than 150 members[28]. These molecular switches fulfill critical roles in physiologic and pathologic events, including regulation of cell proliferation, differentiation, survival, polarity, shape, movement, regulation of gene expression, vesicle transport, and many other activities. An enormous number of upstream activators and regulators of Ras GTPases were indentified. Upon the other side, many different proteins are downstream effectors of activated GTP-bound forms of Ras GTPases. An extensive crosstalk among pathways involving Ras GTPases results in a complex signalling network. Many GPCR-initiated events also activate Ras GTPases further increasing crosstalk.

Activation of GPCRs that couple to other G proteins (*eg.*, Gi) can also promote the PI signalling. PI-PLC β2 and PI-PLC β3 seem to be directly activated by Gβγ dimers released from G proteins. The C terminal domain of PI-PLC β subfamily is necessary for membrane association, Gαq-dependent activation, and promotion of the GTPase activity of Gαq and related α-subunits. Gβγ was supposed to interact with both the PH domain and the catalytic core of PI-PLC β2 and PI-PLC β3. Growth factors promote PI signalling involving receptor autophosphorylation and generation of phosphotyrosine binding sites for PI-PLC γ enzymes on the activated receptor[29,30]. Also small GTPases of the Ras superfamily[31] may activate selected PI-PLC isoforms such as PI-PLC β2, PI-PLC γ2, and PI-PLC ε [32]. PI-PLC β3 also binds Rac, although with lower affinity.

5. Estrogens and PI-PLC

In rat uterus, estrogen receptors probably mediate rapid signaling events[33,34], resulting in modification of transcription[35,36]. Thus, also in this case, the effect of 17β-estradiol depends on the receptors expressed in each tissue. In immature mice, the estrogen receptors erythrocyte sedimentation rate 1 (ESR1) and ESR2, present in uterine stroma and epithelium, differently respond to 17β-estradiol, which increases the expression of ESR1 and decreases the expression of ESR2. 17β-estradiol controls the uterine activities, involving genomic responses, activated by the binding of the estrogen receptors on estrogen-

responsive elements (EREs) present in the promoter region of 17 β-estradiol-regulated genes[37]. This event is biphasic. Within the first hours, hyperemia and uterine imbibition was observed. After 24-72 h, epithelial cells proliferation and differentiation occurs. Furthermore, 17 β-estradiol-mediated regulation of IGF1 transcription and uterine growth involves the direct binding of ESR1 to EREs. 17 β-estradiol responses in the uterus may also occur independently of ERE-mediated transcription, probably involving non classical ESR1 signaling mechanisms, such as protein-to-protein interactions with transcription factors. A non-genomic signaling from membrane ESR1 contributing to 17 β-estradiol-mediated modulation of gene transcription was also described. In mice uteri, GPER1 mediates the rapid signaling cascade. The activation of GPER in epithelial cells enhances negative regulatory actions in the stroma, inhibiting ERK/12 and ESR1 phosphorylation signals, which are essential for the onset of 17 β-estradiol-regulated uterine epithelial cell proliferation[37]. ESR1, ESR2 and GPER mediate rapid signaling events activating different downstream pathways, including the MAPK, phosphatidylinositol 3-kinase and PI-PLC pathways, which in turn modulate transcription. 17 β-estradiol promotes rapid effects on the rat endometrium through activation of PI-PLC mediated PI hydrolysis and requires nucleo-cytoplasmic shuttle of ESR1. GPER probably plays an additive role to this event. Membrane initiated steroid signaling was suggested to be involved in benign and malignant cells of several tissues. These effects occur from seconds to minutes after administration of 17 β-estradiol and involve the rapid activation of many signaling molecules (16, 19, 41), including PI-PLC enzymes. Treatment with 17 β-estradiol induced a rapid increase on total (3H)-inositol phosphate accumulation in the endometrium mediated by PI-PLC activation. Pharmacological inhibition of PI-PLC with the specific inhibitor U73122 decreased the total (3H)-inositol phosphate accumulation. Pre-treatment with the estrogen receptors antagonist ICI 182, 780 blocked the total (3H)-inositol phosphate accumulation induced by 17 β-estradiol. These data indicate that ESR1 acts as an upstream regulator for total(3H)-inositol phosphate accumulation. GPER also seems to contribute to PI-PLC pathway activation in uterus, and mRNA for GPER was detected in the endometrium from rat in estrus. *In vivo* administration of G-1, a selective agonist of GPER, failed to activate expression of Wnt-4, Frizzled-2, IGF-1 or cyclin E1 in uterus[38]. GPER may play a role in uterine physiology, since G-1 and ICI 182, 780[39,40] induced a rapid time-dependent increase in total (3H)-inositol phosphate accumulation in the endometrium. The G-1-mediated effects were blocked by pretreatment of the endometrium with G15 and PI-PLC inhibitors, suggesting that the axis GPER1-PLC is also involved in this intracellular pathway.

6. PI-PLC isoforms in the human endometrium

Many studies were performed to investigate each PI-PLC isoform and the interacting molecules, but their potentialities are far to be highlighted. In our previous studies, we identified a peculiar panel of expressed PI-PLC isoforms in normal human endometrium, namely PI-PLC β 1, β 3, δ 1 and δ 3 isoforms. However, the expression differed in endometriosis tissues with respect to normal endometrium. In women affected with endometriosis, PI-PLC β 1, PI-PLC β 3 and PI-PLC δ 3 were expressed in the eutopic endometrium. PI-PLC β 1, and PI-PLC β 3 were expressed in ectopic tissues. PI-PLC δ 1 was expressed exclusively in the uterosacral ligament localization of the ectopic tissue, indicating that the expression of PI-PLC enzymes differs in the normal endometrium of unaffected woman compared to endometriosis affected patients. The expression levels of PI-PLC β 1 were higher in the normal endometrium. The concentration of PI-PLC β 1 was moderately lower in the eutopic endometrium and much lower in the ectopic tissue. Remarkably, different expression of PI-PLC β 1 was also described during astrocyte activation after lipopolysaccharide (LPS) stimulation[41,42], suggesting its involvement in the cascade of events following inflammatory stimulus. In endometriosis, the PI-PLC β 1 transcript decreases in the ectopic endometrium, probably related to dysregulation of the activity that PI-PLC β 1 plays in the cell cycle control. However, the role of PI-PLC β 1 remains to be elucidated. The levels of PI-PLC β 3 resulted higher in the endometriotic tissue localized in the uterosacral ligament compared to the normal, to the eutopic endometrium and to the ectopic localization in the ovarian cyst. PI-PLC β 3 findings were controversial compared to our previous studies. In fact, down-regulation was described during astrocyte activation after LPS stimulation[8]. PI-PLC δ 1 was down-regulated exclusively in endometriosis. PI-PLC δ 3, expressed in the normal and eutopic endometrium, was not expressed in the ectopic tissues independently from the establishment site. That is an interesting point in that one endometriosis susceptibility locus was localized on chromosome 17 (17q21)[43], a localization consistent with the region where PLCD3 (OMIM *608795), the gene which codifies for PI-PLC δ 3, maps. The endometriosis susceptibility locus on 17q21 might be related to PLCD3 mapping and PI-PLC δ 3 might be involved in the disease.

7. Molecular function and regulation of PI-PLC enzymes expressed in the human endometrium

Literature data lack about the specific role and regulation of PI-PLC β and γ in the human endometrium, although some isoforms belonging to these sub-families resulted expressed. However, the regulation mechanism of some isoforms were analysed in other tissues. Enzymes in the PI-PLC β sub-family share the structural features present in other sub-families, and are characterized by elongated C-terminus about 450 aminoacidic residues long, containing interacting sites for Gq and for other functions such as membrane binding and nuclear localization[17,28]. In mammals, splicing variants for each PI-PLC β isoform were identified. These enzymes are regulated by G proteins with high GTPase stimulating (GAP) activity. Mammalian PI-PLC β enzymes are differentially distributed in tissues. PI-PLC β 1 is the most widely expressed, especially in specific regions of the brain. PI-PLC β 1 exists as alternative splicing variants PI-PLC β 1a and -β 1b, which differ in C-terminal residues[39]. In the cytoplasm, PI-PLC β isoforms function as effectors for receptors belonging to the

rhodopsin superfamily. They are activated by a variety of stimuli and require special combinations of Gα and Gβγ subunits to couple to the effector. Phosphatidic acid (PA) can activate PI-PLC β enzymes, suggesting a role for PA in the regulation of GPCR signal transduction mediated through PI-PLC β1 homodimerization[44]. With the exception of PI-PLC β4, PI-PLC β isozymes are also activated by Gβγ dimmers[45-47]. The relative sensitivity of PI-PLC β enzymes to Gβγ subunits differs from that to Gqα subunits. PI-PLC β1 is the least sensitive to Gβγ[11,45,46]. The Gβγ dimer interacts with PI-PLC β1, β3, and with high affinity PI-PLC β2[48]. Gαq subunit seems to be essential for PI-PLC β activation and cannot be replaced by Gβγ. Two different regions of PI-PLC β interact with Gqα and Gβγ subunits. Male and female Plcb1 knock-out mice exhibited impaired reproductive behaviour. Interestingly, female mice's reduced fertility was probably related to abnormal control of ovulation, whilst the impairment of male mice infertility is probably due to abnormal behaviour. Noteworthy, hormones regulate the reproductive processes through GPCR, which activate a number of enzymes, including PI-PLC β.

PI-PLC δ sub-family is considered the most basic due to its simple structure, and probably was the first to appear in primitive eukaryotes. PI-PLC δ sub-family is the most sensitive to calcium concentrations[49,50]. Tissue transglutaminase Ⅱ (TGⅡ) associates and activates PI-PLC δ1 *in vitro*[51]. TGII has both transglutaminase activity and GTPase activity. Expression of TGⅡ mutants lacking the interaction with α-adrenoreceptor (αAR) or PI-PLC δ1 reduced the increase in intracellular calcium[52], suggesting the involvement of PI-PLC δ1 in αAR signaling. The Rho GTPase-activating protein (RhoGAP) p122 directly activates PI-PLC δ1[53]. Microinjection of the GAP domain of p122 suppressed the formation of stress fibers and focal adhesions induced by lysophosphatidic acid, suggesting a specific GTPase-activating function of p122 for Rho[54]. Therefore, p122 synergistically functions as a RhoGAP and an activator of PI-PLC δ1 *in vivo*, inducing cytoskeletal reorganization.

Plcd1 null mice presented with hair loss associated with abnormal hair follicle structures and epidermal hyperplasia in interfollicle epidermis[55], common findings in skin inflammation. The expression of pro-inflammatory cytokines, such as IL-1β and IL-6, significantly increases and infiltration of immune cells is observed[56]. Therefore, lack of Plcd1 results in induction of skin inflammation, successfully treated with anti-inflammatory reagents. The Plcd1 null mouse was compared to nude mouse[57], in which Foxn1 gene is spontaneously mutated[58], leading to insufficient hair keratin mHa3 expression and abnormal hair shaft structures[59]. Expression of mHa3 was also decreased in the skin of Plcδ1 null mice. In addition, exogenously expressed Foxn1 induced Plcd1 expression. Plcd1 was abundantly expressed in hair follicles of control mice, whereas faint expression was observed in hair follicles of nude mice. These results confirm that Foxn1 acts as an upstream regulator of PI-PLC δ1. On the other hand, PI-PLC δ1 may be involved in the Foxn1/mHa3 axis regulation. Although Plcd3 null mice showed no abnormalities, Plcd1/d3 double null mice resulted in embryonic lethality at E11.5-E13.5 due to defects of placental development[16], including reduction of vessels number in the labyrinth architecture. Interestingly, Plcd1/d3 double null mice embryos supplied with a normal placenta by the tetraploid aggregation method[60] survived beyond E14.5. Placentas of rescued Plcδ1/-δ3 double null mice embryos contained many maternal and embryonic vessels, indicating that the embryonic lethality is caused by a defect in trophoblasts. Therefore, PI-PLC δ1 and PI-PLC δ3 are essential in trophoblast/placenta development. Interestingly, homozygous deletions of the murine chromosome 9 region containing a few genes, including Plcd1, result in phenotype abnormalities, such as alopecia in both genders and male infertility.

PI-PLC δ1 was also suggested to be an anti-oncogene. PLCD1, the human gene which codifies for PI-PLC δ1, maps in 3p22 chromosomal region, which is frequently deleted in esophageal squamous cell carcinoma (ESCC)[61]. Absence of PLCD1 was frequently detected in both primary ESCCs and ESCC cell lines. Introduction of PLCD1 into ESCC cells suppresses the tumorigenic ability. In addition, down-regulation of PI-PLC δ1 protein seems to significantly correlate with ESCC metastasis. The observation of hyperprasia and enhanced proliferation in the skin of Plcd1 null mice suggests that PI-PLC δ1 might play a suppressive role in the development and progression of ESCC. Moreover, DLC-1[62], a gene frequently deleted in liver cancer, shares high homology with the rat p122 RhoGAP[62]. DLC-1 inhibited human cancer cell growth and *in vivo* tumorigenicity in nude mice. Therefore, p122 might function as an anti-oncogene by synergic interaction with PI-PLC δ1 and by modulating the Rho-mediated reorganization of cytoskeleton[63]. PI-PLC δ1 has both nuclear export and import sequences that contribute to its shuttling between the cytoplasm and nucleus[63]. PI-PLC δ1 is generally found in the cytoplasm of quiescent cells, and may also localize in nuclear structures in the G1/S boundary of the cell cycle[64]. Suppression of PI-PLC δ1 by siRNA increases the level of cyclin E, a key regulator of the G1/S boundary, alters S-phase progression, and inhibits cell proliferation[65]. In addition, transient expression of PI-PLC δ1 suppressed the expression of cyclin E at the G1/S boundary. Therefore, PI-PLC δ1 probably plays a role in cell cycle progression.

8. Conclusion

Further studies are required to elucidate the role of PI-PLC isoforms in endometrium, with special regard to the crosstalk among the expressed enzymes. Moreover, the extensive network of interactions with G proteins and oestrogens suggests that the PI-PLC enzymes might act as crucial players during the activities of normal endometrium. However, research efforts will be necessary to highlight the involvement of PI-PLC pathway in endometriosis. In fact, the different expression of PI-PLC enzymes in normal endometrium with respect to endometriotic counterpart and in different localizations of the shed tissue suggests that the PI signal transduction pathway might be related to endometriosis pathogenesis. Our previous studies about the role of PI-PLC in inflammation suggest that the PI transduction system might act a role interfering with the

inflammation cascade widely described in the disease. This interesting hypothesis opens the way to further studies addressed to analyze the activity and regulation of PI-PLC isoforms in the endometrium, and to delineate their relationship with inflammation, representing a promising approach in order to develop novel molecular therapeutic strategies for endometriosis and, probably, for other endometrial diseases. Finally, studies addressed to identify signal transduction pathways interacting upstream and downstream with the PI-PLC system might allow the identification of further molecular targets useful to design novel molecular strategies for therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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