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Review

Regulation of membrane trafficking and endocytosis by protein kinase C: emerging role of the pericentrion, a novel protein kinase C-dependent subset of recycling endosomes

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Abstract. The protein kinase C (PKC) family of isoenzymes has been shown to regulate a variety of cellular processes, including receptor desensitization and internalization, and this has sparked interest in further delineation of the roles of specific isoforms of PKC in membrane trafficking and endocytosis. Recent studies have identified a novel translocation of PKC to a juxtanuclear compartment, the pericentrion, which is distinct from the Golgi complex but epicentered on the centrosome. Sustained activation of PKC (longer than 30 min) also results in sequestration of plasma membrane lipids and proteins to the same compartment, demonstrating a global effect on endocytic trafficking. This review summarizes these studies, particularly focusing on the characterization of the pericentrion as a distinct PKC-dependent subset of recycling endosomes. We also discuss emerging insights into a role for PKC as a central hub in regulating vesicular transport pathways throughout the cell, with implications for a wide range of pathobiologic processes, e.g. diabetes and abnormal neurotransmission or receptor desensitization.

Keywords. PKC, endocytosis, trafficking, pericentrion.

Protein kinase C

The protein kinase C (PKC) family of enzymes consists of 11 isoforms of serine threonine kinases, first identified from bovine cerebellum by the Nishizuka laboratory [1]. These enzymes play important roles in regulation of cell growth, differentiation, apoptosis, secretion, neurotransmission and signal transduction [2, 3].

The PKC family is divided based on sequence and cofactor/activator requirements into three subfamilies: classical/conventional, novel and atypical. All PKC isoenzymes contain a regulatory amino-terminal domain and a catalytic carboxyl-terminal domain. Classical PKCs (cPKCs) require calcium, diacylglycerol (DAG) and an anionic phospholipid (primarily phosphatidylserine) for activation. This family includes PKC alpha, beta, and gamma isoforms, each of which contains two cysteine-rich domains: a phospholipid-binding and DAG/phorbol ester-binding C1 domain, and a calcium-binding C2 domain. Novel PKCs (nPKCs) require DAG but not calcium and thus have a C1 domain without a functional C2 domain; PKC delta, epsilon, eta, nu, and theta belong to this group. Due to the presence of the C1 domain in both these subfamilies, both cPKCs and nPKCs can be activated by the biologically active phorbol esters, which act by mimicking the interaction of endogenous DAG with the enzyme

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[4]. Members of the third subfamily, atypical PKCs, contain a partial/trunctated C1 domain unable to bind DAG or phorbol esters, and thus require neither calcium nor DAG for activation; PKC iota/lambda and zeta isoforms comprise this final group. Below, we will briefly describe current knowledge about signaling through PKC.

PKC in 'classical' signaling

PKC activation and signal transduction

The 'classical' signaling pathway for PKC occurs upon activation of either receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) [3]. Binding of ligands to GPCRs regulates the activity of intracellular GTP-binding proteins (G proteins), some of which lead to activation of the β isoform of phospholipase C (PLC beta). This initiates a cascade of events, starting with the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into the second messengers, inositol 1,4,5-trisphosphate (IP_3) and DAG. IP_3 increases intracellular concentrations of calcium, whereas DAG directly activates cPKCs and nPKCs. Likewise, binding of certain growth factors (including epidermal growth factor and platelet-derived growth factor) and hormones to RTKs leads to tyrosine phosphorylation and activation of PLC gamma, which subsequently results in DAG formation and PKC activation [5].

A number of other interactions can also influence the regulation and localization of PKC isoenzymes. Many PKC isoforms, upon activation, bind to specific proteins (RACK proteins; receptors for activated C kinase) through sites in the C2 domain of activated PKC [6–8]. These RACK proteins may influence the localization of PKC to specific subcellular destinations. In addition, PKCs can be regulated by various lipids other than DAG. Notably, arachidonic acid and related fatty acids have been shown to activate cytosolic PKC in a mechanism distinct from that of DAG [9].

PKC itself can also undergo autophosphorylation, which has been implicated as a mechanism that regulates 'desensitization' of PKC (see below) and was found to be critical for persistent activation and membrane targeting [10]. In addition, several isoforms (such as alpha, delta and beta) can be posttranslationally phosphorylated at serine/threonine residues by phosphatidylinositol-trisphosphate-dependent kinase (PDKs) [2, 11].

The molecular mechanism of PKC activation via DAG is a multistep process. Prior to binding of an activator, PKC exists as an inactive folded structure with a predominant cytosolic localization within the cell. This innate inactivity is due to autoinhibition of the catalytic domain (on the carboxyl-terminal end of the enzyme) by a pseudosubstrate domain (on the amino-terminal end of the enzyme) [3]. The generation of calcium and DAG results in translocation of PKC to the plasma membrane where the C1 and C2 domains can bind with DAG/phosphatidylserine and calcium/anionic phospholipids, respectively. The interaction with DAG also causes the pseudosubstrate domain to separate from the catalytic domain, allowing PKC to interact with its substrates and subsequently initiate PKC-dependent signal transduction processes. Some of these target substrates and downstream signaling pathways include transcription factors (such as IκB/NF-κB, c-Fos and c-Jun), members of the MAP kinase (MAPK) cascades, ion channels and neuroamine transporters such as the norepinephrine transporter (NET) [12] and several transmembrane receptors such as the epidermal growth factor receptor (EGFR) [13], μ-opioid receptor (MOR) [14] and glutamate receptor (GluR2) [15].

PKC inactivation/desensitization

Once the classical model of activation is accomplished within 30–90 seconds following activation of RTKs or GPCRs, several mechanisms appear to operate to ensure acute and long-term termination of PKC signaling.

As far as acute regulation is concerned, recent studies have demonstrated that termination of PKC translocation is a regulated process, which is termed 'reverse translocation'. Following initial translocation to the plasma membrane, PKC isoenzymes return to the cytoplasm in a process that requires catalytic activity of PKC, autophosphorylation of the isoenzymes and interactions with yet to be identified substrate(s)/interacting proteins [10].

Oxidative-related mechanisms have also been shown to inactivate PKC [16]. Hydrogen peroxide and N-chlorosuccinimide were shown to inhibit phorbol diester binding to PKC [17], and site-specific oxidation at the regulatory and/or catalytic domain irreversibly inactivates PKC [2, 11]. The bioactive lipids sphingosine and ceramide have also been shown to regulate PKC activity. Sphingosine has been shown to inhibit PKC *in vitro* and in cells through interference with DAG binding [18]; however, whether endogenously generated sphingosine also carries out this function has not yet been established. On the other hand, ceramide has been shown to indirectly inhibit PKC alpha by enhancing dephosphorylation at multiple sites, including the activating loop site, without inhibiting the ability of PKC alpha to be translocated by phorbol ester [19].

The precise mechanisms for long-term downregulation and degradation of PKC are much less certain, although a number of mechanisms have been suggested and partially evaluated. PKCs are substrates for the calpains, a class of cysteine proteases, at least *in vitro*. Additionally, some PKC isoforms, i.e. PKC alpha and epsilon, can be downregulated by the ubiquitin/proteasome-mediated degradative pathway [20, 21]. In that regard, all PKC isoforms (except delta) express PEST sequences, segments

that help target proteins for degradation by the proteasome [22].

Role of PKC in endocytic trafficking

Once activated, PKC regulates numerous cellular functions [3]. In particular, multiple studies have defined roles for PKC in the regulation of receptor desensitization and internalization, and emerging recent evidence points to more general roles of PKC in regulating the processes of endocytosis and trafficking. This section presents a general discussion of endocytosis and some of the new possibilities for roles of PKC in regulating endocytic trafficking.

Endocytosis and vesicular trafficking

Endocytosis can be broadly defined as the uptake of extracellular materials and can be divided into three types: phagocytosis (cell eating), pinocytosis (cell drinking) and regulated endocytosis [13]. Regulated endocytosis imports larger substances (such as proteins) after they have been bound to a cell surface receptor. This mode of endocytosis is particularly relevant for signal transduction as it is involved in transcellular delivery, protein destruction and modulation of hormone/growth factor responses.

Endocytosis is currently appreciated to occur via clathrin-dependent and clathrin-independent mechanisms [23, 24]. In the clathrin-dependent pathway, endocytosis typically occurs at sites in the plasma membrane that are enriched with the trimeric protein clathrin. Many receptors are found on pit-like indentations of the cell membrane; upon membrane invagination, clathrin coats the pits and pinches them off to form new vesicles. After internalization of the extracellular material, the vesicles lose the clathrin coat and fuse with endosomes.

A variety of lipids and proteins – including signaling receptors and glycosphingolipids – undergo endocytosis without the help of these clathrin-coated pits [24]. The molecular details of the clathrin-independent pathway may involve another group of plasma membrane invaginations (caveoli) that have not yet been fully characterized [25]. A very recent study showed that clathrin-independent endocytosis is blocked by depletion of sphingolipids [26]. That study also delineated three specific subsets of clathrin-independent endocytosis (caveolar, RhoA and Cdc42 dependent, respectively) with unique requirements for sphingolipids. While caveolar-dependent endocytosis was shown to be glycosphingolipid dependent, the RhoA- and Cdc-42-dependent pathways seem to require sphingomyelin.

Both the clathrin- and caveoli-dependent pathways require the GTPase dynamin for internalization and further endocytosis. Interestingly, dynamin-independent endocytosis has also been reported [27].

Once internalized, cell surface receptors begin their traffic through the endosomal compartments [28]. First, endocytic vesicles uncoat and fuse with early (sorting) endosomes, resulting in delivery of the cell surface receptor cargo. Next, depending on the cargo's ultimate fate, some molecules recycle back to the plasma membrane, while other molecules, targeted for degradation, accumulate in the late endosomes. Molecules return back to the cell surface along two different routes: a Rab4-dependent fast recycling or a Rab11-regulated slow recycling route. Late endosomes, often referred to as prelysosomal compartments because they serve as transient repositories along the route to lysosomes, are characterized by the presence of active proteolytic enzymes and low internal pH. Although some proteolysis takes place in the late endosomes, complete degradation occurs in lysosomes.

Emerging roles of PKC in endocytic trafficking

PKC has been implicated as an important regulator of endocytosis and trafficking of various recycling molecules. Although the precise mechanisms are still unclear, PKC appears to function as a central hub in controlling vesicular pathways of receptors, transporters, channels and plasma membrane proteins [29, 30].

One of the key functions in which PKC has been implicated relates to membrane receptor downregulation. Previous studies have shown that phorbol esters induce receptor phosphorylation, interaction of PKC with the receptors (perhaps forming a 'receptor/PKC complex') and, at times, receptor degradation [2].

It is now generally accepted that following ligand binding, GPCR-stimulated increases in calcium and DAG lead to activation of PKC. Activated PKC plays an important role in GPCR signaling by mediating the phosphorylation of downstream proteins. Additionally, PKC also phosphorylates serine and threonine residues within cytoplasmic loops and C-terminal domains of many GPCRs [31, 32], triggering their desensitization and/or endocytosis [33]. Binding of beta arrestins, adapter proteins that can serve as signal terminators, enhances this process of endocytosis for many GPCRs [33, 34]. Internalized receptors are recycled back to the plasma membrane through either a 'fast recycling' pathway (primarily for class A GPCRs) or they may reside for extended periods of time in endosomes before undergoing 'slow recycling' or degradation (primarily for class B GPCRs) [23, 33]. Several bodies of evidence suggest pivotal roles for PKC in regulating both of these pathways of GPCR trafficking [14, 35–42].

PKC also exerts significant effects on trafficking and internalization of growth factor receptors such as c-Met and the EGFR. In the case of c-Met, PKC was shown to control not only microtubule-based trafficking of the

receptor, but also to promote accumulation of c-Met in perinuclear compartments [43]. Recent studies also suggest that activation of PKC leads to direct phosphorylation of EGFR at a single threonine residue (Thr654). This phenomenon was shown to decrease the affinity of ligands for the receptor [44], as well as to reduce receptor kinase activity [45]. More importantly, PKC-mediated threonine phosphorylation of the EGFR appears to regulate its intracellular trafficking. Evidence indicates that the phosphorylation of Thr654 diverts internalized EGFR from a ubiquitin-mediated degradative fate into a recycling pathway [46].

In addition to the regulation of receptor trafficking, a limited but increasing literature points to significant regulation of trafficking of transporters, channels and plasma membrane proteins by PKC. These include the dopamine transporter [47], norepinephrine transporter [48], serotonin transporter [49], glutamate transporter [50], hCAT-1 [51], epithelial Na^+ channels [52], Kv4 channels [53], ROMK1 channels [54], integrins [55] and E-cadherins [56], with the list continuing to grow as more PKC-dependent mechanisms become elucidated.

The dopamine transporter (DAT), in particular, has garnered attention as being downregulated during acute PKC activation by redistribution of DAT from the plasma membrane to endosomal compartments [47, 57–59]. Thereafter, PKC was also shown to mediate phosphorylation and trafficking of the dopamine receptors D_2 [60] and D_1 [61]. The mechanism of transporter downregulation was shown to involve both increased DAT internalization as well as decreased DAT recycling, with both effects being mediated primarily through actions of PKC [47]. On the other hand, Miranda et al. [62] demonstrated PKC-dependent ubiquitination of DAT, which may target DAT for lysosomal degradation. This ubiquitination was shown to be important for interaction of DAT with adapter proteins in clathrin-coated pits [63].

Jayanthi et al. [12, 48] showed that phosphorylation and lipid raft-mediated internalization of another transporter, NET, is also PKC dependent. The same group showed that the serotonin transporter (SERT) is also regulated by PKC. This regulation occurs in a biphasic manner that involves PKC-dependent phosphorylation of the transporter on different residues [49].

Another example of PKC-mediated effects involves PKC-dependent targeting of synaptotagmin IX to the recycling compartment [64]. Recent studies have also implicated PKC in the internalization of a number of viruses (rhabdovirus, adenovirus, influenza, and herpes simplex virus), albeit through different forms of endocytosis [65].

PKC-dependent sequestration of molecules to the perinuclear recycling compartment, the 'pericentrion'

Recent studies have led to the identification of a novel site of translocation of a subset of PKC isoenzymes, findings which have implications for the regulation of endocytosis and availability of proteins and lipids at the plasma membrane. Upon sustained stimulation of PKC by phorbol esters (30–60 min), two PKC isoforms, alpha and betaII, were shown to translocate to a region adjacent to the nucleus [29]. The resultant subcellular compartment was found to be close to, but distinct from, the Golgi complex, and in close proximity to the centrosome; therefore, it has been dubbed the 'pericentrion' [29, 30, 66]. PKC isoforms alpha and betaII internalized in what appeared to be endocytic vesicles migrating on tubular tracks. Indeed formation of the pericentrion was shown to be dependent on intact microtubules, in that treatment with the microtubule depolymerizing agent nocodazole inhibited translocation from plasma membrane to the pericentrion. In contrast, the actin poison cytochalasin D did not inhibit internalization to the pericentrion but led, rather, to some dispersal of this compartment once it was formed.

Interestingly, sequestered PKC colocalized with Rab11, a marker of the perinuclear recycling compartment. However, whereas Rab11 remained resident in the pericentriolar region, PKC displayed dynamic migration to and from this compartment, dependent upon continued activation of PKC. Moreover, activation of PKC also caused accumulation of internalized transferrin to the same compartment, demonstrating a general effect on recycling endosomes [29]. Therefore, the pericentrion is functionally defined as the PKC-dependent subset of recycling endosomes formed by the relocation of endocytic vesicles to the Rab11-positive perinuclear region.

Importantly, sustained activation of PKC also led to sequestration of several membrane proteins to the pericentrion, including caveolin and CD59. PKC stimulation also led to accumulation of plasma membrane lipids, such as the GM1 gangliosides, in the pericentrion [66]. In contrast, components and markers of the endolysosomal compartment did not sequester to the pericentrion upon PKC stimulation.

Functionally, it was also shown that pericentrion formation and sequestration of PKC requires clathrin-dependent endocytosis. Interestingly, molecules that are known to be internalized through clathrin-independent pathways were also sequestered into this region upon PKC activation. These results suggest that the/a 'site' of PKC action in the internalization process may be distal to initial events at the plasma membrane, and may be at the level of 'destination' of cargo to either recycling, lysosomes, or sequestration (Fig. 1).

Further studies of this novel effect of PKC revealed that pericentrion formation displays a strict temperature requirement and does not occur below 32 °C [66]. A very recent study on PKC-dependent regulation of ligand-gated ion channels demonstrated a similar temperature dependence [67]. This feature is quite distinct from general endocytosis, which is known to be inhibited below 16 °C. Importantly, the temperature effect was reversible and dynamic: translocation of PKC at 37 °C was reversed with subsequent incubation at 4 °C. Further reincubation at 37 °C restored translocation of PKC to the pericentrion

Formation of the pericentrion also requires persistent activation of PKC. Thus, once the pericentrion is formed, treatment with a PKC inhibitor causes dispersal of this compartment. This finding supports the notion that the formation of the pericentrion is a dynamic and reversible process.

Mechanistically, translocation of PKC to the pericentrion was shown to be dependent on phospholipase D (PLD). Inhibitors of PLD, dominant negative mutants of PLD [30], and siRNA directed against PLD1 and PLD2 inhibited this process. Moreover, PLD itself was shown to localize in the perinuclear region, were it is thought to interact with translocated PKC [68].

Whereas it was noted that phorbol myristate acetate (PMA)-induced pericentrion formation was observed in many cell lines (e.g. HEK293, HeLa, A549, HUVEC), it failed to occur in MCF-7 breast cancer cells. Further studies showed that PMA treatment leads to generation of ceramide from the salvage pathway in this cell line [30], whereby breakdown of complex sphingolipids (including sphingomyelin) results in formation of sphingosine and resynthesis of ceramide. It was then shown that ceramide can negatively regulate pericentrion formation, such that specific inhibition of this pathway restored PKC-dependent translocation in MCF7 cells. Moreover, exogenous and endogenous ceramide was shown previously to inhibit endocytosis [69]. Thus, formation of the pericentrion may be mediated by a delicate balance between PKC and ceramide.

Conclusions, implications and future directions

It is becoming increasingly appreciated that PKC regulates the internalization and trafficking of many plasma membrane receptors, transporters and other proteins, often resulting in receptor desensitization and/or 'removal' of those proteins from the plasma membrane. Mechanistic investigations, for the most part, have focused on the specific effects of PKC on individual targets, often implicating direct phosphorylation of the target proteins by PKC as a potential mechanism for their downregulation. On the other hand, the recent observations on the effects of PKC on endocytosis and formation of the pericentrion, suggest an alternative/additional mechanism by which sustained activation of PKC may result in simultaneous sequestration of many recycling components.

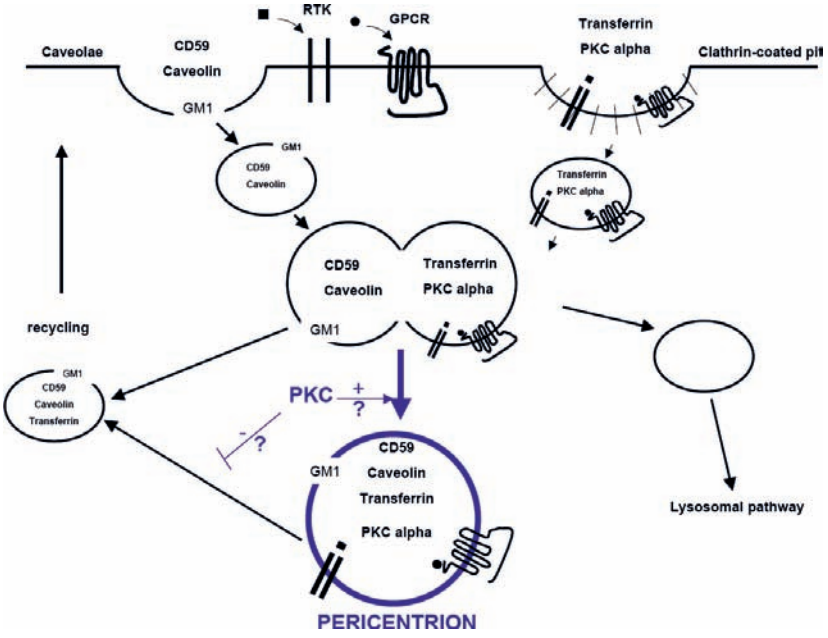

Figure 1. Model of PKC-mediated pericentrion formation. Sustained stimulation of PKC leads to translocation of several plasma membrane proteins and lipids, i.e. CD59, caveolin, GM1, transferrin and PKC alpha. One may also speculate that PKC-dependent desensitization and downregulation of RTKs and GPCRs involve similar perinuclear accumulation. This figure is modified and reproduced with permission from Idkowiak-Baldys et al. [66].

As such, sustained stimulation of PKC may occur as a result of activation of PLD and/or sustained action of ligands on their receptors, which, in turn, results in the PKC-dependent targeting of endosomes to the perinuclear region.

Such a mechanism may have implications for the actions of hormones and neurotransmitters. Prolonged activation of PKC in response to these extracellular agents would provide a powerful mechanism for sustained desensitization, through trapping of the receptors in the pericentrion. Such effects can have substantial consequences for biology and pathobiology. For example, the effects of PKC on the serotonin/dopamine receptors may have implications for anxiety [70], depression [71] bipolar disorders [72] and other disorders involving these receptors and transporters. PKC may also be involved in trapping of other transporters, although the intracellular location where these proteins are trapped remains to be elucidated. For example, effects of PKC on trafficking of the Glut4 transporter may have profound effects on the pathogenesis of diabetes mellitus, as it has been shown that persistent hyperglycemia results in sustained activation of PKC. It is tempting to speculate that PKC-dependent formation of the pericentrion and trapping of key glucose regulatory proteins may relate to previous studies implying a role for PKC (especially PKC beta) in the pathogenesis of diabetic complications [73]. On the other hand, atypical isoforms of PKC are implicated in regulating Glut4 translocation to the plasma membrane upon insulin stimulation [74]. Thus, sustained activation of classical and inhibition of atypical isoforms of PKC could lead to insulin-resistant diabetes by affecting Glut4 trafficking.

Thus, although numerous studies support critical roles for PKC in trafficking of specific proteins, the exact mechanisms governed by PKC remain poorly understood. Specifically, there is a need to define the events following PKC activation that lead to the internalization and sequestration of cell surface proteins and related molecules. The requirement for both PKC and PLD activities suggests a role for bioactive lipids such as phosphatidic acid, in addition to DAG, in this process. Additionally, defining the pericentrion as a distinct PKC-dependent subset of recycling endosomes could be a conceptual stepping stone to a better understanding of cellular trafficking on a broader level. Finally, understanding how PKC regulates endocytosis and/or trafficking is likely to be of particular importance in pathological processes that are caused by abnormal changes in trafficking of various signaling molecules.

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