Review

Protein kinase C isoforms and lipid second messengers: a critical nuclear partnership?

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Summary. A growing body of evidence, accumulated over the past 15 years, has highlighted that the protein kinase C family of isozymes is capable of translocating to the nucleus or is resident within the nucleus. The comprehension of protein kinase C isoform regulation within this organelle is under development. At present, it is emerging that lipid second messengers may play at least two roles in the control of nuclear protein kinase C: on one side they serve as chemical attractants, on the other they directly modulate the activity of specific isoforms.

One of the best characterized lipid second messenger that could be involved in the regulation of nuclear PKC activity is DAG. The existence of two separate pools of nuclear DAG suggests that this lipid second messenger might be involved in distinct pathways that lead to different cell responses.

Nuclear phosphatidylglycerol, D-3 phosphorylated inositol lipids and nuclear fatty acids are involved in a striking variety of critical biological functions which may act by specific PKC activation.

The fine tuning of PKC regulation in cells subjected to proliferating or differentiating stimuli, might prove to be of great interest also for cancer therapy, given the fact that PKC-dependent signaling pathways are increasingly being seen as possible pharmacological target in some forms of neoplastic diseases.

In this article, we review the current knowledge about lipid second messengers that are involved in regulating the translocation and/or the activity of different protein kinase C isoforms identified at the nuclear level.

Key words: Lipid second messengers, Protein kinase C, Nucleus, Diacylglycerol, Phospholipase

Introduction

Twelve serine/threonine protein kinases constitute the protein kinase C (PKC) family which transduces a myriad of signals implicated in a multitude of physiological processes (Hug and Sarre, 1993). Three distinct subfamilies of PKC isoforms can be identified according to their dependency on three combinations of activators: conventional PKCs (α, βI, βII, γ) require phosphatidylserine (PS), diacylglycerol (DAG), and Ca2+; novel PKCs (δ, ε, η, θ) need PS and DAG but not Ca2+; atypical PKCs (z, i/λ, µ) are insensitive to both DAG and Ca2+ (Hug and Sarre, 1993; Newton, 1997). Analysis of the primary structure of PKC reveals the presence of four domains conserved across PKC isoforms (C1-C4) and five variable domains that are divergent (V1-V5). Two functional domains have been described in PKC: an amino terminal regulatory domain and a carboxyl terminal catalytic domain (Hug and Sarre, 1993). The catalytic domain (V3-V5) contains the ATP binding site and is thought to interact with the substrates (Hug and Sarre, 1993). Fatty acids, D3-phosphorylated inositol lipids, phosphatidic acid (PA), and ceramide are additional molecules that are considered to be capable of activating PKC (Newton, 1997; Liu and Heckman, 1998).

Over the last years, many reports indicated that PKC isoforms can translocate to the nucleus or are resident
within this organelle (for a review see Olson et al., 1993; Martelli et al., 1999a).

In this review, we shall address the issue of how these isoforms are recruited and activated by lipid cofactors. In particular, we will examine the nuclear lipid activators (i.e. DAG, phosphatidylglycerol, D3-phosphorylated inositol lipids, free fatty acids) that interact with PKC to regulate its function in this organelle.

Nuclear DAG

One the best characterized lipid second messenger that could be involved in the regulation of nuclear PKC activity is DAG. Approximately 50 different DAG species have been identified in mammalian cells, due to the fact that the fatty acid at the sn-1 position of DAG is saturated or mono-unsaturated, whereas the sn-2 fatty acid position is more variable (Hodgkin et al., 1998). At least three pathways can generate DAG: a) phosphatidylinositol (PtdIns) (4,5)P₂ is hydrolyzed through the action of a phosphoinositide-specific phospholipase C (PI-PLC) generating DAG and inositol(1,4,5)P₃; b) phosphatidylcholine (PC) is hydrolyzed by a phospholipase D (PLD), yielding PA, which in turn is converted to DAG by a specific PA phosphohydrolase; c) PC is hydrolyzed by a PC-specific phospholipase C (PC-PLC) which produces DAG (Perry and Hannun, 1998). It has been shown that when DAG derives from inositol lipid hydrolysis it is polyunsaturated. On the contrary, DAG deriving from PC is mono-unsaturated/disaturated (Hodgkin et al., 1998). If DAG originates through the activation of PI-PLC isozymes, its increase is rapid and transient, whereas if DAG is produced from PC its rise is more sustained (Hodgkin et al., 1998; Wakelam, 1998).

The involvement of nuclear DAG, derived from PtdIns(4,5)P₂ hydrolysis, in attracting and/or activating PKC to the nucleus was first hypothesized in insulin-like growth factor-I (IGF-I)-stimulated Swiss 3T3 cells (Divecha et al., 1991). This hypothesis was substantiated by the finding that IGF-I activated the nuclear PI-PLC-β1 isoform (Martelli et al., 1992) and by the data showing that only a specific inhibitor of PI-PLC (ET-18-OCH₃), but not inhibitors of either PLD or PC-PLC, blocked in Swiss 3T3 cells both the IGF-I-dependent rise in nuclear DAG and migration to the nucleus of PKC-α (Neri et al., 1998). These results, obtained in our laboratory, were in agreement with those by Sun et al. (1997), who demonstrated that in HL-60 cells there is an increase in nuclear DAG during the G2/M phase transition. The use of ET-18-OCH₃, D609, and propranolol (selective phospholipase inhibitors) allowed the authors to conclude that DAG derived from inositol lipid hydrolysis and that it was responsible for intranuclear translocation of PKC-β1 isoform. The papers of Neri et al. (1998) and Sun et al. (1997) are very important because they demonstrated for the first time DAG derived from PtdIns(4,5)P₂ hydrolysis as the PKC partner responsible for its translocation to the nucleus.

Similar conclusions were subsequently reached by other investigators who studied nuclear DAG generation in insulin-stimulated NIH 3T3 cells (Martelli et al., 2000), Interleukin 2-treated human Natural Killer cells (Vitale et al., 2001) or in platelet-activating factor-stimulated nuclei from rat liver cells (Miguel et al., 2001). There is no indication, however, of the PKC isoforms (if any) that translocate to the nucleus in these experimental models.

Other reports focused the attention on nuclear DAG levels derived from the PC pool. In IIC9 fibroblasts, α-thrombin stimulation induced PKC-α translocation to the nucleus. Kinetic analysis indicated that, after 3 min of stimulation, nuclear DAG levels increased four-fold and also showed a ten-fold increase in nuclear PKC-α activity (Leach et al., 1992; Jarpe et al., 1994). The same group, by means of capillary gas chromatography, demonstrated that the molecular species profiles of the nuclear DAG resembled that of PC and not inositol lipids at all times of stimulation (Jarpe et al., 1994). All these findings pointed to the likelihood that a PLD activity was responsible for the increase in nuclear DAG mass. Unexpectedly, however, it was subsequently reported that the generation of nuclear DAG in response to α-thrombin stimulation of IIC9 cells was insensitive to ethanol and therefore was not due to activation of a PLD, but rather of a nuclear PC-PLC (Baldassare et al., 1997).

D’Santos et al. (1999) came to a similar conclusion when analyzing the DAG molecular species of murine erythroleukemia cell nuclei. They hydrolyzed nuclear inositol lipids or PC with specific phospholipases and the DAG generated was phosphorylated to form PA, using a non-specific DAG kinase, in the presence of [γ-32P]ATP. PA was then dimethylated and separated taking advantage of argentation chromatography which allows distinction of different molecular species on the basis of double bond number. Nuclear inositol lipid-derived DAG was predominantly unsaturated, whilst that derived from nuclear PC was predominantly mono-unsaturated. The analysis of total nuclear DAG showed it derived mostly from PC and that more than 90% of total species were disaturated or mono-unsaturated.

They also observed by argentation chromatography that the DAG generated in vitro during a 30 °C incubation of isolated nuclei was highly mono-unsaturated. This in vitro DAG generation was insensitive to D609 (a supposedly selective inhibitor of PC-PLC), ET-18-OCH₃ (which reportedly inhibits PI-PLC), U73122 (an inhibitor of PI-PLC) or butanol. Intact cells treated with butanol did not show either a decrease in the mass of nuclear DAG or an accumulation of phosphatidylbutanol in the nucleus, suggesting that, if a PLD activity was present, butanol could not act as a substitute for water in the transphosphatidylation reaction.
Jones et al. (2002) came to similar conclusions in T-lymphocytes, suggesting the existence of a major pool consisting of saturated or monosaturated fatty acids derived from PC, whereas the minor pool consisted of more unsaturated species, most likely derived from phosphoinositides. Their data strongly argued for the presence of a nuclear PC-PLC activity in agreement with recent findings by others who indicated in PC the source of nuclear DAG in nuclei of phorbol ester-treated neuroblastoma cells (Antony et al., 2001).

A PLD-mediated pathway for generating DAG has been identified in nuclei from Madin-Darby canine kidney cells that displayed an accumulation of PA prior to or during an ethanol-sensitive DAG formation (Balboa et al., 1995; Balboa and Insel, 1995). Taken together, the data coming from different laboratories were consistent with the idea that nuclei may contain two separate pools of DAG, which are independently regulated to produce structurally distinct molecular species of DAG. In some cases, changes in nuclear DAG were detectable only if nuclei retained their envelope (Leach et al., 1992; Jarpe et al., 1994; Baldassare et al., 1997), whereas in others no variations of DAG mass could be measured if the membrane was present (e.g. Divecha et al., 1991).

The existence of two separate pools of nuclear DAG suggests that this lipid second messenger might be involved in distinct pathways that lead to different cell responses. However, a conclusive demonstration that in the same cell line different stimuli activate distinct phospholipases present in the nucleus and that this differential activation is responsible for attracting to the organelle-specific, DAG-dependent PKC isozymes, is still lacking.

We very recently provided evidence that in the HL-60 cell line, employing nuclei retaining the envelope, nuclear PI-PLC activity causes changes in DAG levels after a proliferating stimulus represented by IGF-I, and that this increase in DAG mass is responsible for PKC-βII translocation to the nucleus. In contrast, in response to DMSO administration (i.e. a differentiating stimulus), we observed a rise in nuclear DAG levels and a translocation of PKC-α to the nucleus, which were blocked by inhibitors selective for PLD-mediated DAG generation. Therefore, we can now postulate the existence in the cell nucleus of two independently-regulated DAG sources, related to distinct stimuli and capable of recruiting to the nucleus different PKC isoforms (Neri et al., 2002).

We would also like to mention that there are some reports in which an increase in nuclear DAG has been related to the translocation of PKC isozymes to the nucleus, but the source of nuclear DAG is unknown. For example, Ritz et al. (1993) reported a rise in nuclear rat liver DAG levels, in response to heat stress, which was concomitant with a stimulation of nuclear (but not cytosolic) PKC activity. A rise in nuclear DAG and PKC activity has been demonstrated to occur during the S-phase in regenerating rat liver, at 16-24 h after hepatectomy (Banfic et al., 1993). Other papers showed the activation of a nuclear PI-PLC over a similar time frame in regenerating rat liver (Kuriki et al., 1992; Neri et al., 1997). It should be emphasized that there is also a report hinting at the stimulation of a nuclear PLD activity, which was followed by intranuclear migration of PKC-δ (Banno et al. 1997). This might be another example of a cell type in which there is an activation of two distinct phospholipases in the nucleus.

Finally, a very rapid rise, peaking at 1 min of stimulation, in nuclear DAG and PKC-βII was demonstrated to occur in erythropoietin-stimulated B6Sut. EP cells, a subclone of a previously established murine bone marrow stem cell line (Mallia et al., 1997). In Table 1 we have summarized the biological systems in which changes in nuclear DAG mass and PKC activity have been reported.

### Table 1. Source of nuclear DAG and translocated PKC isoforms.

<table>
<thead>
<tr>
<th>DAG SOURCE</th>
<th>CELL LINE/TISSUE</th>
<th>PKC ISOFORMS</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol lipids</td>
<td>Swiss 3T3</td>
<td>α</td>
<td>Neri et al., 1998</td>
</tr>
<tr>
<td>Inositol lipids</td>
<td>HL-60</td>
<td>βII</td>
<td>Sun et al., 1997; Neri et al., 2002</td>
</tr>
<tr>
<td>Inositol lipids</td>
<td>NIH 3T3</td>
<td>unknown</td>
<td>Martelli et al., 2000</td>
</tr>
<tr>
<td>Inositol lipids</td>
<td>human Natural Killer cells</td>
<td>unknown</td>
<td>Vitale et al., 2001</td>
</tr>
<tr>
<td>Inositol lipids AND PC</td>
<td>murine erythroleukemia</td>
<td>unknown</td>
<td>D'Santos et al., 1999</td>
</tr>
<tr>
<td>Inositol lipids AND PC</td>
<td>CTLT-2</td>
<td>Unknown</td>
<td>Jones et al., 2002</td>
</tr>
<tr>
<td>PC</td>
<td>HL-60</td>
<td>α</td>
<td>Neri et al., 2002</td>
</tr>
<tr>
<td>PC</td>
<td>IIC9</td>
<td>α</td>
<td>Leach et al., 1992; Jarpe et al., 1994; Baldassare et al., 1997</td>
</tr>
<tr>
<td>PC</td>
<td>Madin-Darby</td>
<td>unknown</td>
<td>Balboa et al., 1995; Balboa and Insel, 1995</td>
</tr>
<tr>
<td>PC</td>
<td>LA-N-1</td>
<td>unknown</td>
<td>Antony et al., 2001</td>
</tr>
<tr>
<td>Unknown</td>
<td>rat liver</td>
<td>unknown</td>
<td>Banfic et al., 1993</td>
</tr>
<tr>
<td>Unknown (PC ?)</td>
<td>rat liver</td>
<td>δ (?)</td>
<td>Banno et al., 1997</td>
</tr>
<tr>
<td>Unknown</td>
<td>B6Sut.EP</td>
<td>βII</td>
<td>Mallia et al., 1997</td>
</tr>
</tbody>
</table>

**Nuclear phosphatidylglycerol**

The PKC-βII isozyme selectively translocates to the nuclear envelope in HL-60 cells in response to proliferative stimuli, leading to the phosphorylation of lamin B (Goss et al., 1994). Extraction of nuclear
envelopes with either non-ionic detergents or organic solvents abolished the in vitro PKC-βII phosphorylation of lamin B. The phosphorylation was reconstituted by adding nuclear membrane extracts, indicating the existence of a βII PKC-selective nuclear membrane factor (NMAF) that stimulated the activity of PKC-βII on histone H1. Since NMAF activity was resistant to exhaustive protease treatment, the authors hypothesized a nuclear envelope lipid or lipid metabolite (Murray et al., 1994). A subsequent study revealed NMAF to be phosphatidylglycerol (PG) capable of stimulating in vitro PKC-βII activity, which was dependent on both the glycerol head group and fatty acid side chain composition.

Starting from the previous observation that PG binds selectively and saturably the carboxyl terminal region of PKC-βII (Murray and Fields, 1998), Gokmen-Polar and Fields (1998) demonstrated that PG-mediated activation of this PKC isoform involves interaction only with the carboxyl terminal 13 amino acids of the protein (-βII V5). The authors proposed that PG coclusters with PS into Ca2+-dependent microdomains, whose formation is induced by PKC and which possibly bind the C2 region of the kinase.

If DAG is also present, PKC activation occurs, through the displacement of the pseudosubstrate domain from the active site of the enzyme (Gokmen-Polar and Fields, 1998). An interaction of V5 region with the C2 region of active PKC has indeed been hypothesized by others (Keranen and Newton, 1997).

**Nuclear D-3 phosphorylated inositol lipids**

Phosphorylation of the 3-position of the inositol ring of polyphosphoinositides is catalyzed by members of the family of phosphoinositide 3-kinases (PI 3-Ks) (Rameh and Cantley, 1999). To date, nine members of the PI 3-K family have been isolated and cloned from mammalian cells, and they are classified into three classes on the basis of the substrates that they preferentially utilize (Domin and Waterfield, 1997). PI 3-K members can generate four different lipid products: PtdIns(3)P, PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(3,4,5)P3. These are thought to act by themselves as second messengers and are involved in a striking variety of critical biological functions (Rameh and Cantley, 1999).

The activity of some PKC isozymes (the novel -δ, -ε, -η isotypes, and the atypical -ζ form) can be controlled through PI 3-K (Toker and Cantley, 1997). In vivo, PI 3-K is both necessary and sufficient to activate PKC-ζ. However, in vivo PKC-ζ is phosphorylated and activated by a polyphosphoinositide-dependent kinase (PDK-1) which phosphorylates threonine 410 in the PKC-ζ phosphorylation loop. Evidence coming from in vitro experiments showed PDK-1 to phosphorylate and activate PKC-ζ in a PtdIns(3,4,5)P3-enhanced manner (Chou et al., 1998). It has been proposed that PDK-1 and PKC-ζ exist as an inactive complex in cells. Upon agonist stimulation, the complex is likely to be recruited at the sites of PtdIns(3,4,5)P3 production. Here, PDK-1, and, possibly, PKC-ζ, bind PtdIns(3,4,5)P3-ensuing in the phosphorylation of threonine 410 and activation of PKC-ζ (Le Good et al., 1998). A similar model of activation has been proposed for PKC-δ (Le Good et al., 1998). Interestingly, the nuclear translocation of PI 3-K in NGF-treated PC12 cells, was accompanied by translocation and activation of nuclear PKC-ζ (Neri et al., 1994, 1999a).

**Nuclear fatty acids**

The stimulation of PKC by fatty acids has been well characterized (Khan et al., 1995). For example, saturated fatty acids that have carbon chain lengths of C13 to C18 activate PKC-γ and -ε in vitro (Kasahara and Kikkawa, 1995). Cis-unsaturated fatty acids such as oleate and arachidonate, synergize with DAG and PS in activating PKC and in some cases the oleate-dependent activation of PKC has been reported to be independent of both PS and Ca2+ (Khan et al., 1995; Shinomura et al., 1991). It seems that fatty acids interact with PKC at a site distinct from the phorbol ester/DAG binding site (Khan et al., 1995). Fatty acids are released from the sn-2 position of phospholipids through the action of phospholipase A2 (PLA2) (Khan et al., 1995).

Nuclei reportedly contain various types of PLA2, which have been demonstrated to be present either in the nuclear interior (bound to the nuclear matrix) or in the nuclear envelope. Interestingly, it seems that a nuclear location of PLA2 is a characteristic of rapidly dividing cells (see Fayard et al., 1998 and references therein). A possible link between nuclear PKC and fatty acid production has been provided by Freeman et al. (1998) who reported that angiotensin II was capable of inducing a nuclear translocation of PLA2 in vascular smooth muscle cells, with a subsequent release and accumulation of arachidonic acid within the nucleus after 10 min. Interestingly, a short (12 min) exposure to angiotensin II caused in these cells the intranuclear migration of PKC-α (Haller et al., 1994), a phenomenon that might be related to the production of arachidonic acid in the nucleus.

**Concluding remarks**

The knowledge about regulation of nuclear PKC isoforms is rapidly growing, even if there are many gaps to be filled in. As discussed above, there is strong evidence about a pivotal role played by DAG in regulating some PKC isoforms at the nuclear level. However, much less information is available regarding DAG-independent isoforms.

In addition, the role played by phosphorylation of PKC isoforms should not be overlooked. It is now established that serine phosphorylation at a conserved carboxyl-terminal motif of some PKC isoforms is very important to transform the kinase into the mature, cofactor-responsive enzyme. PKC must be processed by three distinct phosphorylation events before it is competent to respond to second messengers.
(e.g. Keranen et al., 1995; Tsutakawa et al., 1995). As far as PKC-α is concerned, its autophosphorylation at Ser-657 controls the accumulation of phosphate at other sites on the kinase, as well as contributing to the maintenance of the phosphatase-resistant conformation (Bornancin and Parker, 1997). Regarding PKC-β II, Ser-660 phosphorylation causes a 10-fold increase in the enzyme’s affinity for PS and Ca²⁺ (Edwards and Newton, 1997). For the first time, we demonstrated that PKC isoforms which migrated to the nucleus are phosphorylated (Neri et al., 2002).

Further studies, employing molecular biology approaches (such as overexpression of PKC isoforms mutated in regions which are critical for lipid binding, or ablation of enzymes that are important for the generation of intranuclear lipid second messengers, see Neri et al., 1999b; Martelli et al., 1999b), biochemical and pharmacological approaches (synthesizing potent mimics of lipid second messengers that activate PKC isoforms) should lead to a further level of comprehension in the study of regulation of nuclear PKC by lipid signaling molecules.

Overall, we feel that the definition of very early intranuclear events that are critical for the attraction and the regulation of PKC isoforms in the nucleus is of fundamental importance.

The fine tuning of PKC regulation, as we have recently demonstrated in HL-60 cells (Neri et al., 2002) subjected to proliferating or differentiating stimuli, might prove to be of great interest also for cancer therapy, given the fact that PKC-dependent signaling pathways are increasingly being seen as a pharmacological target in some forms of neoplastic diseases (Parker, 1999; Watters and Parsons, 1999).

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