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Review

Nuclear protein kinase C isoforms: key players in multiple cell functions?

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Summary. Protein kinase C (PKC) isozymes are a family of serine/threonine protein kinases categorized into three subfamilies: classical, novel, and atypical. PKC isozymes, whose expression is cell type-specific and developmentally regulated, are key transducers in many agonist-induced signaling cascades. To date at least 10 different PKC isotypes have been identified and are believed to play distinct regulatory roles. PKC isoforms are catalytically activated by several lipid cofactors, including diacylglycerol. PKC is thought to reside in the cytoplasm in an inactive conformation and to translocate to the plasma membrane or cytoplasmic organelles upon cell activation by different stimuli. However, a sizable body of evidence collected over the last 15 years has shown PKC to be capable of translocating to the nucleus. Furthermore, PKC isoforms can reside within the nucleus. Studies from independent laboratories have to led to the identification of several nuclear proteins which act as PKC substrates as well as to the characterization of some nuclear PKC-binding proteins which may be of fundamental importance for finely tuning PKC function in this peculiar cell microenvironment. Most likely, nuclear PKC isozymes are involved in the regulation of several important biological processes such as cell proliferation and differentiation, neoplastic transformation, and apoptosis. In this review, we shall summarize the most intriguing evidence about the roles played by nuclear PKC isozymes.

Key words: Signal transduction, Phosphorylation, Proliferation, Differentiation, Apoptosis

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Introduction

PKC is a multigene family of serine/threonine protein kinases involved in the transduction of a bewildering number of signals important for the regulation of cell growth, differentiation, apoptosis, and a plethora of other functions (Hug and Sarre, 1993; Nishizuka, 1995; Dempsey et al., 2000; Musashi et al., 2000). At present 10 PKC isoforms have been cloned. They have been classified into three main groups that share a common requirement for phospholipid for their activity, but differ in structure and dependencies on other activators. Conventional PKCs (cPKCs), -α, -ßI, -ßII, -γ, meet the original definition of PKC as a Ca^{2+}- and phospholipid-dependent protein kinase (Nishizuka, 1995). Indeed, they require phosphatidylserine (PS), Ca^{2+}, and diacylglycerol (DAG) or phorbol esters; novel PKCs (nPKC), -δ, -ε, -η, -θ, require only DAG and PS; and atypical PKCs (αPKCs), -ζ, -ι/-λ , are dependent on PS. (Hug and Sarre, 1993; Dempsey et al., 2000; Musashi et al., 2000). The structure of PKC isozymes includes conserved regions (C1-C4) that are interrupted by variable regions (V1-V5). Each isoform contains a catalytic and a regulatory domain (Hug and Sarre, 1993).The catalytic domain can be active in the absence of cofactors after proteolytic removal of the regulatory domain by cleavage in the V3 region. The regulatory domain, which is responsible for dependence on cofactors, contains an autoinhibitory pseudosubstrate region as well as sequences that mediate the interactions of PKC with phospholipids, DAG/phorbol esters, and specific anchoring proteins. The Ca^{2+} dependency is mediated by the C2 region (which is indeed absent in nPKCs), while phorbol ester binding requires the presence of two cysteine-rich zinc-finger regions within the C1 domain. aPKCs lack one of the two cysteine-rich zinc-finger regions and therefore do not bind (and cannot be activated by) phorbol esters. (Hug and Sarre, 1993; Dempsey et al., 2000; Musashi et al., 2000).

The biological functions of PKC have mostly been linked with events occurring at the plasma membrane

level and/or in the cytoplasm, because PKC isotypes are thought to be associated with the cytoskeleton in an inactive state and, after maturation (phosphorylation), they translocate to the plasma membrane (or the membrane of cytoplasmic organelles) to become fully activated in the presence of specific cofactors (Hug and Sarre, 1993; Newton, 1997). However, increasing evidence has implied a role for PKC in nuclear functions, suggesting this may be a pathway to communicate signals generated at the plasma membrane to the nucleus (Olson et al., 1993; Buchner, 1995). Almost every PKC isoform has been reported to be present in the cell nucleus in some cellular system under certain conditions, either as the consequence of translocation from cytoplasm or as a resident enzyme.

Here, we will briefly overview the literature supporting a role for lipid second messengers in the attraction of some PKC isotypes to the nuclear compartment. Then, we shall review the emerging details on mechanisms regulating nuclear import of PKC, as well as the fine subnuclear distribution of different PKC isoforms, including their interactions with other nuclear components. Moreover, we will address the issue of nuclear proteins which serve as substrates for PKC isozymes. Finally, we shall summarize the biological responses which appear to be mediated, at least in part, by nuclear PKCs, such as cell proliferation, differentiation, neoplastic transformation, and apoptosis.

Nuclear lipid second messengers and PKC isoforms

A key issue is whether PKC isoforms translocate to the nucleus as a consequence of nuclear lipid signaling (Neri et al., 2002a). Although this is not easy to assess, there are some strong clues supporting such a theory. In Swiss 3T3 cells treated with insulin-like growth factor 1 (IGF-1), nuclear DAG increases and PKC-α migrates to the nucleus (Divecha et al., 1991). If the IGF-1-evoked increase in nuclear DAG was inhibited by the selective phosphoinositide-specific phospholipase C (PI-PLC) inhibitor 1-O-octadeyl-2-O-methyl-sn-glycero-3-phosphocholine, there was no translocation of PKC-α to the nucleus (Neri et al., 1998). Similar results have been reported for HL60 (Sun et al., 1997; Neri et al., 2002b) or U937 human leukemia cells (Deacon et al., 2002), albeit in this case the involved isozyme was PKC-ßII. Thus, it has been proposed that nuclear DAG is the attracting force that drives to the nucleus DAG-dependent cPKCs. An unresolved issue is how PKC isoforms located in the cytoplasm "sense" DAG generated in the nucleus. It has been proposed that nuclear DAG could "trap" PKC during a transient visit to the nucleus (Buchner, 1995). However, this would entail a rapid and continuous cycling of PKC in and out of the nucleus, and therefore seems unlikely because a massive migration of PKC isozymes to the nuclear compartment usually takes place in a short time after stimulation (e.g. Divecha et al., 1991). The question then arises as to what lipid messenger(s) might attract nPKCs or aPKCs to the nucleus. Phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P3), one of the products of phosphoinositide 3-kinase (P3K), has been proposed to act as the driving force for the migration of PKC-ζ to the nucleus in either nerve growth factor (NGF) -treated PC12 cells (Neri et al., 1999) or in C2-ceramide-challenged rat hepatocytes (Calcerrada et al., 2002). Indeed, if generation of PtdIns(3,4,5)P3 in the nucleus by PI3K was blocked by selective pharmacological inhibitors of PI3K (Ly294002 or wortmannin) there was no nuclear translocation of PKC-ζ.

Another fundamental issue that needs to be considered is that serine/threonine phosphorylation at a conserved carboxyl-terminus motif of PKC is very important to transform the kinase into the mature, cofactor-responsive enzyme. All PKC isozymes, except for aPKCs and PKC-δ, must first be processed by three distinct phosphorylation events before they are competent to respond to activators (e.g. Keranen and Newton, 1997; Newton, 2001; Shirai and Saito, 2002). For example, phosphorylation of PKC-ßII on serine 660 causes a ten-fold increase in the enzyme's affinity for PS and Ca^{2+} (Edwards and Newton, 1997). Consistently, phosphorylation of nuclear PKC-α and PKC-ßII has been reported by us (Neri et al., 2002b) and by others (Chiarini et al., 2002).

Nuclear import of PKC

All the proteins that translocate to the nucleus must pass through the nuclear pore complex (NPC), which serves as a gateway through the nuclear envelope. Passage through the NPC is highly regulated by receptor-transporters that selectively bind short regions of amino acids termed the nuclear localization sequence (NLS) (Jans et al., 2000). Two classical NLS that have been described are a lysine-rich amino acid sequence observed in the SV40 large T antigen and a bipartite amino acid sequence of two basic regions separated by a spacer region (Jans et al., 2000). All classes of NLS are believed to be recognized specifically by the α/ß1-importin heterodimer. However, the signal-mediated nuclear transport is also mediated by an array of importin ß isoforms (Jans et al., 2000). Interestingly, importin-ß binds to arginine-rich NLS, whereas, importin-α binds lysine-rich NLS-containing proteins.

PKC-δ possesses a basic NLS between amino acids 611 and 623 (DeVries et al., 2002) that closely resembles the functional bipartite NLS of Myo-D (Vandromme et al., 1995). When mutations were made within this sequence of PKC–δ, nuclear localization of the kinase was significantly inhibited. Both KRK (611-613) and KKK (619, 621, and 623) were required for nuclear import as neither half could drive translocation independently (DeVries et al., 2002). It is very interesting that sequence alignment of cPKCs, nPKCs, and aPKCs showed that PKC–δ NLS is highly conserved in this kinase family.

However, aPKCλ has been shown to contain a

functional bipartite, arginine-rich NLS within the amino-terminus part of the zinc-finger domain (Perander et al., 2001). The exposure of this NLS is regulated by intramolecular interactions between the amino-terminus part of the kinase, including the pseudosubstrate sequence, and the catalytic domain. In addition, aPKC-λ possesses a leucine-rich nuclear export signal in the linker region (amino acids 248-255) between the zinc-finger and the catalytic domain (Perander et al., 2001).

There is an additional level of regulation, however. Phosphorylation of tyrosine 256 (Tyr256) facilitates nuclear import of aPKC-ζ. Tyr256 is located at the lip of the activation loop and is conserved among members of the aPKC group. Upon NGF treatment of PC12 cells, Tyr256 is rapidly phosphorylated prior to aPKC-ζ entry into the nucleus (White et al., 2002). Tyr256 phosphorylation enhanced binding of aPKC-ζ to ß-importin (but not to importin-α) and the binding was abrogated when Tyr256 was mutated to phenylalanine. It was thus proposed that phosphorylation of aPKC-ζ at Tyr256 induces a conformation, whereby the NLS is exposed, which then binds importin-ß leading to import of the kinase into the nucleus (White et al., 2000).

Is an interaction with importins always required for PKC entry into the nucleus? It does not seem so, because the phorbol ester-induced transport of PKC-α was not affected by microinjection of antibodies against importin-ß or of non-hydrolyzable GTP analogues (Schmalz et al., 1998). In addition, nuclear import of PKC-α could not be inhibited by wheat germ agglutinin or an antibody to nuclear pore-complex protein (Schmalz et al., 1998). The issue of whether or not PKC-α possesses a functional NLS is highly controversial. A sequence analysis showed that PKC-α has only three out of the six amino acids which constitute the NLS of PKC−δ (DeVries et al., 2002). An important factor which regulates PKC-α entry into the nucleus seems to be cytoskeletal integrity, because if the cytoskeleton is disrupted by cytochalasin B or colchicine, phorbol ester-elicited nuclear translocation of PKC-α is inhibited. In contrast, the nuclear accumulation of NLS-containing reporter protein in an in vitro nuclear transport assay was not affected by these drugs (Schmalz et al., 1996).

Altogether, the data summarized above indicate that investigation of the mechanisms regulating nuclear import of PKC isoforms is a very intriguing area of research.

Subnuclear localization of PKC

The most obvious localization of PKC in the nucleus would be the nuclear envelope. Indeed, there are reports showing translocation of PKC-α to the nuclear envelope of NIH 3T3 cells stimulated with phorbol esters (Leach et al., 1989), or of PKC-ßII to the nuclear membrane of K562 human erythroleukemia cells in response to bryostatin (Hocevar et al., 1992). Nevertheless, numerous studies have shown that PKC could move from the cytoplasm further inside the nucleus. For example, in quiescent Swiss 3T3 fibroblasts IGF-1 caused an increase in PKC-α within the nucleus, except for nucleoli, as demonstrated by confocal laser scanning microscopy analysis (Neri et al., 1994). The presence of PKC-α inside the nucleus could allow the enzyme to phosphorylate not only proteins located near the nuclear envelope but also chromatin and/or nuclear matrix proteins. When several PKC isozymes are simultaneously present within the nucleus, each one may have its own distinct localization, as suggested by a very detailed immunocytochemical investigation performed in nervous NG-108-15 cells (Beckmann et al., 1994). It was indeed shown that the -α isozyme was located in the nuclear interior, excluding nucleoli, whereas the -δ isotype was only found in nucleoli. PKC-ε was localized in the pore complexes at the nuclear envelope. In this context, an issue that should be considered is that contradictory findings have in some cases been reported concerning the nuclear localization of PKC isoforms, as revealed by immunocytochemical methods, in a given tissue or cell line (Newton, 1997). The discrepancies may depend, among other things, on the use of antibodies that are not entirely specific. In light of the aforementioned problems, alternative techniques to analyze the subcellular distribution of PKC isozymes would appear desirable. Recently, new approaches to analyze the spatial distribution of PKC isotypes, such as fluorescence resonance energy transfer, have been employed (Bastiaens and Jovin, 1996). At present, the advent of jellyfish-green fluorescent protein (GFP) technology provides unprecedented opportunities to study protein localization in living cells and circumvents problems such as fixation, or inadequate antibody penetration. Chimeric proteins of GFP with several distinct target proteins (located also in the nucleus) have shown their usefulness for localization and trafficking studies in a variety of living cells (e.g. Ogawa et al., 1995; Olson et al., 1995; Rizzuto et al., 1995; Broers et al., 1999). PKC-GFP hybrids have been employed to analyze intranuclear presence of the -ξ isotype in HL60 cells (Bertolaso et al., 1998) or of PKC-α in NIH3T3 cells exposed to phorbol esters (Wagner et al., 2000).

The fine nuclear localization of some PKC isotypes has also been investigated by means of electron microscope/quantitative immunogold labeling. In Swiss 3T3 mouse fibroblasts mitogenically stimulated by IGF-1, PKC-α which, in untreated cells, was mainly present in the cytoplasm, was reduced in this compartment after the stimulus and almost doubled in the nucleus. PKC-α was found not only associated with the nuclear envelope but mainly with the interchromatin domains. By using in situ nuclear matrix preparations, PKC-α appeared to be retained both at the nuclear lamina and at the inner nuclear matrix (Zini et al., 1995). Also PKC-ζ, when analyzed by means of immunoelectron microscopy, was localized to the inner nuclear matrix of PC12 cells (Wooten et al., 1997). The nuclear matrix is viewed by several investigators as the fundamental organizing principle of the nucleus where many functions take

place, including DNA replication, gene expression, and protein phosphorylation (Nickerson, 2001; Berezney, 2002; Martelli et al., 2002a). Several enzymes of the inositol lipid metabolism have been found associated with the nuclear matrix (reviewed in Maraldi et al., 1999; Divecha et al., 2000) and this indicates that the matrix may also be involved in intranuclear lipid signal transduction pathways.

Nuclear-PKC-binding proteins

PKC isozymes display only limited differences in substrate specificity or sensitivity to activators. Since there are usually various isozymes within a given cell type, the differential subcellular localization has been proposed to explain isoform specificity (Ron and Kazanietz, 1999). The differential localization appears to depend, at least in part, on interactions of each PKC isozyme with specific anchoring proteins, referred to as PKC-binding proteins. Over the last 10 years, several PKC-binding proteins have been identified, including the two focal contact proteins, vinculin and talin (Hyatt et al., 1994; Liao et al., 1994; Chapline et al., 1996; Izumy et al., 1997; Chapline et al., 1998). These proteins are referred to as STICKs (substrates interacting with C-kinase), RICKs (receptors for inactive C-kinase), or RACKs (receptors for activated C-kinase) (Mochly-Rosen and Gordon, 1998; Jaken and Parker, 2000). All STICKs are phospholipid-binding proteins and, in general, are localized to interfaces between membranes and cytoskeletal structures (Ron and Mochly-Rosen, 1995). RICKs do not need to be susbstrates for PKC. RACKs are proteins that directly interact with the C2 domain of PKC. RACK1 is the major anchoring protein for PKC-ßII (even though it also binds the -α and -ε isotypes), while RACK-2 is the PKC-ε specific RACK (Ron and Mochly-Rosen, 1995; Ron et al., 1995). These anchoring proteins are thought to direct PKC isoforms to various cell compartments such as focal contacts, the Golgi apparatus, caveolae, and elements of the cytoskeleton. Interestingly, RACK1 is up-regulated in angiogenesis and human carcinomas (Berns et al., 2000), while ethanol treatment induces uncoupling of PKC-ßII from RACK1 (Ron et al., 2000). Elucidation of PKC-RACK interactions allowed the synthesis of PKC translocation inhibitors and activators (Johnson et al., 1996; Yedovitzky et al., 1997). These isozyme-selective translocation inhibitors and activators are of potential therapeutic value (Dorn et al., 1999). Our laboratory was the first to identify a few nuclear PKC-binding proteins of Swiss 3T3 cells by means of overlay assays with recombinant PKC-α (Martelli et al., 2000). The proteins turned out to be lamin A/C and B, as well as nucleolin. All these proteins are substrates for PKC (Martelli et al., 1999), so that they can be considered STICKs. Recently, we have further characterized the molecular interactions between PKC-α and both lamin A and B (Martelli et al., 2002b; Tabellini et al., 2002) by showing that a portion of the C2 region of the kinase is essential for binding either lamin A or B. Specificity of the interaction seems to depend on the V5 region for lamin A, whereas for lamin B the V1 region is involved. Other nuclear PKC-α-binding proteins have been identified by means of overlay assays: PTB-associated splicing factor (PSF); p68 RNA helicase; and the heterogeneous nuclear ribonucleoprotein (hnRNP) proteins A3 and L (Rosenberger et al., 2002). The interaction between PSF and PKC-α could also be demonstrated in a glutathione-S-transferase (GST)-pull-down assay, using GST-PKC-α expressed in insect cells. This finding demonstrated that the interaction between PKC-α and PSF takes place in solution and might be an indication that the two proteins are capable of interacting in vivo. PSF was found to be a weak substrate for PKC-α.

All the nuclear PKC-α anchoring proteins identified by Rosenberger et al. (2002) interact with RNA and are involved in splicing. Therefore, the authors tested a possible involvement of PKC itself in splicing, also because phosphorylation affects splicing. Nevertheless, they were unable to demonstrate a change in either the splicing efficacy or the preferential splice site in the alternative splicing of src.

Nuclear PKC substrates

There is no doubt that the key for understanding the roles played by nuclear PKC isozymes is the identification of their substrates and of the changes caused by phosphorylation. Over the years, a considerable number of nuclear proteins have been identified which are in vivo and/or in vitro substrates for PKC isozymes. These proteins include: histones (H1, H2B, H4), lamins (A/C and B), DNA topoisomerase I and IIα, DNA polymerase α and ß, DNA methyltransferase, polyADP-ribose polymerase, B23/nucleophosmin, CREB, FOS, myogenin, vitamin D3 receptor, BZLF1 (a member of the extended AP-1 family of transcription factors), RNA polymerase II, hnRNP A1 and K, PSF, C23/nucleolin, and p53 (Buchner, 1995; Ron and Kazanietz, 1999; Schullery et al., 1999; Buchner, 2000). Thus, it seems that nuclear PKC isozymes might be involved in the control of DNA replication, mRNA synthesis and processing, nucleo-cytoplasmic transport, and chromatin structure. Nevertheless, there are problems in interpreting these results. Is not clear whether or not PKC-dependent phosphorylation of some of these substrates (for example p53) actually takes place within the nucleus or elsewhere in the cell. Phosphorylation has been demonstrated to alter the properties of some of these substrates (lamin B, DNA topoisomerase I, myogenin, the vitamin D3 receptor, hnRNP A1), therefore presenting a biological relevance (Buchner, 1995), even though the functional consequences of these phosphorylative events are far from being understood. There are, however, two notable exceptions. The first one is represented by phosphorylation of nuclear lamin B, which is a fundamental step for nuclear disassembly

during both G2/M transition of the cell cycle and the execution phase of apoptosis (see later). The second one is the PKC-α-dependent phosphorylation of nuclear PI-PLCß1 on Serine887 in response to IGF-1 stimulation of Swiss 3T3 cells (Xu et al., 2001). This phosphorylation inhibits nuclear PI-PLCß1 activity.

Since some of PKC substrates are involved in mRNA transcription and metabolism, nuclear PKC isoforms might be involved in the control of gene expression. There are indeed some clues that PKC may regulate opioid gene transcription in myocardial cells, although the molecular mechanisms of this regulation are still unclear (Ventura et al., 1997, 1998, 2000; Ventura and Maioli, 2001). Very recent results point to the likelihood that the control might be achieved through dynorphin B-dependent activation of two cardiac lineage-promoting genes, GATA-4 and Nkx-2.5 (Ventura et al., 2003).

Involvement of nculear PKC isozymes in cell proliferation

The most direct and convincing evidence for a proliferation-linked function of nuclear PKC has come from a series of extensive and elegant studies on lamin B phosphorylation carried out in HL60 cells. Lamin B is rapidly phosphorylated by the -ßII isoform of PKC following its translocation to the nucleus after treatment with bryostatin (Hocevar and Fields, 1991), an activator of PKC, or in response to a physiologically relevant stimulus represented by platelet-derived growth factor (Fields et al., 1990). The sites of PKC-mediated phosphorylation lie within the carboxyl-terminus domain of lamin B immediately adjacent to the central α-helical rod domain. Functionally, the phosphorylation of these sites led to the time-dependent solubilization of lamin B. Depletion or inhibition of p34cdc2 kinase activity had no effect on PKC-mediated lamin B phosphorylation (Hocevar et al., 1993). Since lamin B phosphorylation occurs during the nuclear envelope breakdown at the G2/M transition, the possibility existed that it was due to different kinases and not only to p34cdc2/cyclin B kinase, as previously thought (Moir et al., 1995 and references therein). Indeed, it was susbsequently demonstrated that both p34cdc2/cyclin B kinase and PKC-ßII could phosphorylate purified soluble lamin B. However, PKC-ßII phosphorylated interphase lamin B at more than 200 hundred times the rate of p34cdc2 kinase. Moreover, it was found that PKC-ßII translocated to the nucleus during the G2/M transition, concomitantly with the phosphorylation of the -ßII site Serine405 of lamin B. This site is not a target for p34cdc2/cyclin B kinase, thus indicating a physiological role for nuclear PKC-ßII in mitotic lamin phosphorylation (Goss et al., 1994). Additional studies demonstrated that HL60 cells could be arrested in G2 phase by exposure to the selective PKC pharmacological inhibitor, chelerythrine chloride. The involvement of -ßII PKC in the G2/M transition was demonstrated by three lines of evidence. Firstly, chelerythrine caused a dose-dependent inhibition of PKC-ßII, similar to that for G2 phase blockade in whole cells. Secondly, it specifically inhibited PKC-ßII-mediated lamin B phosphorylation and mitotic nuclear lamina disassembly. Thirdly, chelerythrine-mediated G2 phase arrest resulted from selective inhibition and degradation of PKC-ßII (Thompson and Fields, 1996). Taken together, these findings pointed to the relevance that PKC-ßII activation is required for nuclear lamin B phosphorylation and disassembly and entry into mitosis. The same group have tried to identify a specific activator of nuclear PKC-ßII at the G2/M transition. They have demonstrated that during the G2/M phase, in parallel with increased phosphorylation of lamin B, there was a striking increase in the levels of nuclear DAG, capable of stimulating nuclear PKC activity in vitro (Sun et al., 1997). Moreover, they have found that specificity of translocation of PKC-ßII to the nuclear envelope and of lamin B phosphorylation could be largely attributed to the presence of phosphatidylglycerol (PG), a remarkable observation that led to the discovery of another player in nuclear lipid signaling (Murray et al., 1994; Murray and Fields, 1998) (Fig. 1). PG was capable of selectively stimulating PKC- II kinase activity three/four-fold above the level obtained with optimal concentration of DAG, Ca^{2+}, and PS. The target of PG is the carboxyl-terminus region of PKC-ßII. It should be emphasized, however, that the main function of nuclear PG conceivably resides in modulating nuclear PKC-ßII translocation. In fact, at variance with nuclear DAG, nuclear PG levels do not change appreciably during the cycle of HL60 cells.

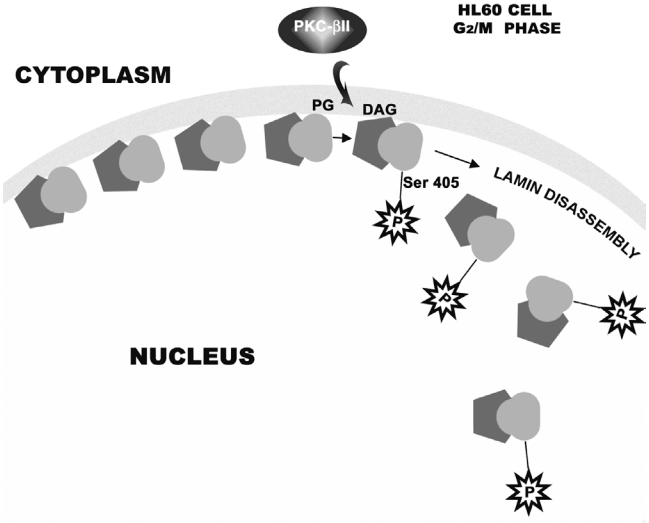

Fig. 1. PKC-ßII phosphorylates lamin B during the Gß2/M transition in HL60 cells. Cytoplasmic PKC-ßII is attracted to the nuclear envelope and activated by a combination of DAG and PG. While the levels of nuclear envelope DAG increase during the G2/M transition, those of PG do not. PKC-ßII phosphorylates lamin B at Serine405 (Ser 405) and this results in disassembly of the nuclear lamin followed by nuclear envelope solubilization. It is worth remembering that HL60 cells do not express lamins A/C (Paulin-Levasseur and Julien, 1992).

Therefore, it is possible that PG serves primarily to facilitate and/or enhance the selective binding of PKC-ßII to the nuclear envelope where it can be fully activated in the presence of elevated DAG generated during G2 phase (Murray and Fields, 1998).

Then, there is a plethora of studies, performed in a variety of cell lines treated with a multitude of mitogenic stimuli, which are highly suggestive of an important role played by PKC isotypes in the control of cell proliferation (reviewed in Buchner, 1995; Martelli et al., 1999; Buchner, 2000). In most of these articles, the authors showed that one or more PKC isozymes translocated to the nucleus in response to agonist stimulation. Presumably, this is an indication of an important role played by PKC within the nucleus. Nevertheless, none of these investigations has directly addressed the issue of the substrates and/or the events that could be affected by nuclear PKC.

Nuclear PKC and the control of cell differentiation

Similarly to cell proliferation, there is a sizable body of evidence which suggests that increased nuclear levels of different PKC isoforms play an important role in the mechanisms which regulate the differentiation of cells, mainly of hematolymphopoietic lineage (reviewed in Martelli et al., 1999). All these investigations showed a translocation of various PKC isoforms from cytoplasm to nucleus in response to differentiating stimuli. However, also in this case, there is a lack of functional studies which have addressed the issue of how PKC, once in the nucleus, may influence cell differentiation. The only study which convincingly demonstrated the importance of nuclear PKC in determining cell differentiation is that by Pierce et al. (1998), who showed that an overexpressed, constitutively activated form of PKC-α, was localized primarily within the nucleus of hematopoietic progenitor cells and caused macrophage development even in the presence of stimuli that normally promote only neutrophilic development. Since they found that macrophage colony-stimulating factor promoted translocation of PKC-α to the nucleus, they concluded that this is a nuclear signal associated with macrophage development in primary mammalian hematopoietic progenitor cells. Nuclear PKC-α might also be involved in the differentiation of mouse erythroleukemia cells. Indeed, it translocates to the nucleus in response to hexamethylen-bis-acetamide (HMBA), and HMBA-dependent erythroid differentiation was blocked in cells overexpressing an antisense RNA for PKC-α (Mallia et al., 1999). A connection between nuclear PKC, gene expression and differentiation may be represented by PKC-dependent phosphorylation of transcription factors with ensuing changes in their DNA-binding or regulatory properties. However, there is only a single paper in which a firm link was established between PKC translocation to the nucleus, cell differentiation and phosphorylation of a nuclear substrate. In NGF-treated PC12 cells, PKC-ζ rapidly migrates within the nucleus where it phosphorylates 106-kDa C23/nucleolin (Zhou et al., 1997). Nucleolin is not a transcription factor but rather a multifunctional nucleolar phosphoprotein implicated in chromatin structure, rDNA transcription, rRNA processing, ribosome assembly and maturation, and nucleo-cytoplasmic transport (Ginisty et al., 1999). All these events may be important for cell differentiation. Nevertheless, it is still unclear what the functional consequences of the PKC-ζ-dependent phosphorylation of nucleolin are.

Nuclear PKC and neoplastic transformation

Most of the interest that has initially surrounded PKC stemmed from the identification of this enzyme as the high affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (Hug and Sarre, 1993; Nishizuka, 1995). Thus, it is not surprising that several groups have tried to address the issue of whether or not nuclear PKC isoforms may be implicated in carcinogenesis. Once again, these studies suffer from the lack of a clear relationship between the two events investigated: neoplastic transformation and nuclear localization of PKC isoforms, even though some results are very intriguing. For example, Cd^{2+} (an established carcinogen) was found to enhance the effect of phorbol ester on nuclear binding and activation of PKC in mouse 3T3/10 T1/2 fibroblasts. Furthermore, in a reconstituted in vitro system, Cd2+ stimulated PKC binding to a 105-kDa nuclear protein. Interestingly, this 105-kDa protein might correspond to nucleolin, a PKC substrate (see above) (Beyersmann et al., 1994). It was hypothesized that $Cδ2^{+}$ substitutes for Zn2+ in the regulatory domain of PKC, thus rendering the putative protein-protein site exposed.

In diethylnitrosamine-induced hepatocarcinogenesis, both the expression and the activity of PKC-ß were maximal at 60 days post-hepatectomy. As a result, a role for nuclear PKC-ß in promoting the selective growth of carcinogen-initiated hepatocytes in rat liver has been suggested (La Porta and Comolli, 1994). Moreover, using the same experimental model, increased nuclear PKC-ß activity and expression were detected in lung metastatic nodules, suggesting a possible role played by this isoform in the development of secondary tumors as well (La Porta et al., 1997).

In a previous section of this article, we have reviewed the compelling evidence that links PKC-ßII to cell proliferation. It is very intriguing that PKC-ßII could induce cancer in vivo in an organ (the colon) in which it was overexpressed transgenically (Murray et al., 19999 and that this isoform specifically increases in chemically-induced colon cancers (Gökmen-Polar et al., 2001). Nevertheless, although these findings did provide a circumstantial case for PKC-ßII being important for carcinogenesis in vivo, they did not show whether or not its ability to migrate to the nucleus is a crucial factor in this action.

Nuclear PKC and apoptosis

Apoptosis is a highly regulated process leading to cell death and is essential for morphogenesis, development, differentiation, and homeostasis of multicellular organisms. It is largely conserved through evolution and may occur in response to a broad range of stimuli. Its significance is underscored by its occurrence in all higher eukaryotes and by malformations resulting from disturbances of the apoptotic process during development (Vaux and Korsmeyer, 1999). Increased rates of apoptosis are responsible for several degenerative diseases, and inhibition of apoptosis is implicated in autoimmune processes and carcinogenesis (Reed, 2000).

The activation of genetically-controlled, specific pathways results in the characteristic morphological features of apoptosis, that are mainly evident in the nucleus. These include chromatin condensation, nuclear shrinkage and disintegration, and the formation of apoptotic bodies (Robertson et al., 2000). However, apoptosis is also characterized by biochemical changes such as DNA and RNA cleavage, post-translational modifications of proteins, and proteolysis of several polypeptides (e.g. the nuclear lamins). A central biochemical mechanism that leads to apoptotic cell death is the activation of a family of cysteinyl-aspartate-specific proteases referred to as caspases (Patel et al., 1996; Thornberry and Lazebnik, 1998; Utz and Anderson, 2000). Most of the proteins cleaved by caspases have important functions in the cell, so that their cleavage during early stages of apoptosis may be expected to silence basic cellular processes and to lead to disintegration of cell architecture. There is now evidence that phosphorylation of nuclear proteins occurs during apoptosis and that it could facilitate subsequent proteolytic cleavage by caspases (reviewed in Martelli et al., 2001).

The first report hinting at a possible involvement of nuclear PKC in apoptosis demonstrated a redistribution of the kinase toward the nucleus in dexamethasone-treated thymocytes (Trubiani et al., 1994). A direct link between PKC and apoptosis was subsequently provided by Shimizu et al. (1998). In HL60 cells induced to apoptosis by camptothecin, PKC-α phosphorylated lamin B one hour after the addition of the drug and this phenomenon preceded both lamin proteolytic degradation and DNA fragmentation. An important role for PKC-α was supported by several lines of evidence: a) camptothecin stimulated PKC-α activity in HL60 cells; b) both the PKC inhibitor UCN-01 and an anti-PKC antibody prevented lamin B phosphorylation; c) lamin B phosphorylation was restored by recombinant PKC-α; and d) if PKC-α was immunodepleted, phosphorylation of lamin B was reduced by about 90%. We do not know, however, whether or not, in response to camptothecin, there was a translocation of PKC-α from cytoplasm to the nucleus of HL60, even though this seems likely. These results have been questioned by another group which showed that in HL60 cells exposed to the apoptotic inducer cytarabine, the -ζ isozyme of PKC migrated to the nucleus where it co-localized with lamin B (Cross et al., 2000). Moreover, proteolytic activation of PKC-δ by caspase-3 was concomitant with lamin B phosphorylation and proteolysis, while inhibition of PKC-δ by the selective inhibitor, rottlerin, delayed lamin proteolysis. Taking into account that PKC-δ was also capable of phosphorylating lamin B in vitro, it was concluded that this PKC isoform is an apoptotic lamin kinase and that efficient nuclear lamina disassembly at apoptosis requires lamin B phosphorylation followed by its caspase-3-mediated proteolytic degradation. A catalytically active fragment of PKC-δ is generated by proteolysis in cells induced to undergo apoptosis in response to some, but not all, inducers of apoptotic cell death (Kikkawa et al., 2002; Brodie and Blumberg, 2003). The cleavage is presumably effected by caspase 3 between aspartic acid 327 and asparagine 328. This cleavage site of PKC-δ is located between two tyrosine residues (311 and 332) that can be phosphorylated. Phosphorylation of Tyr311 promotes degradation of PKC-δ presumably after ubiquitination (Kikkawa et al., 2002). PKC−δ phosphorylated at Tyr311 may be insensitive to proteolysis by caspase. The catalytic fragment and the tyrosine phosphorylated active form of PKC−δ seem to contribute to promotion of apoptosis independently (Kikkawa et al., 2002).

A key role played by nuclear PKC-δ during apoptosis has also been shown in C5 rat parotid salivary cells incubated with etoposide (DeVries et al., 2002). Indeed, both the caspase-3-generated, catalytic fragment of endogenous PKC-δ, and the full-length endogenous PKC-δ preferentially accumulated in the nucleus of C5 cells in response to δ catalytic fragment resulted in nuclear localization and apoptosis. The import of the catalytic fragment of PKC-δ was dependent on a novel NLS located in the carboxyl-terminus. Importantly, mutations within the NLS prevented nuclear accumulation of the catalytic fragment and apoptosis. An important role for PKC-δ in apoptosis, is also indicated by the fact that it translocates to the nucleus of insulin-secreting RIN1046-38 treated with free fatty acids. Apoptosis induced by this treatment was accompanied by lamin B disassembly that followed PKC-δ migration to the nucleus. Lamin B disassembly and apoptosis were decreased by cell transfection with a dominant negative mutant form of PKC-δ (Eitel et al., 2003). It is worth remembering here that at present great interest surrounds the failure of pancreatic ß-cells induced by elevated free fatty acid levels, because it may be one of the causes leading to type II diabetes (Unger and Orci, 2002).

Nuclear translocation and activation of PKC-δ have also been reported for etoposide-treated C6 glioma cells (Blass et al., 2002) or Jurkat T cells exposed to ionizing radiation, even though in the latter case their relevance in the context of the apoptotic process was not investigated (Cataldi et al., 2002).

All in all, these findings indicate that PKC-δ regulates a or some nuclear event(s) required for apoptotic cell death. Is the role of nuclear PKC-δ in apoptosis restricted to the phosphorylation of lamin B? Most likely, the answer is that it is not. Indeed, results by DeVries et al. (2002) suggest that once in the nucleus PKC-δ can regulate the cytosolic apoptotic machinery, conceivably by affecting the expression of key apoptotic molecules which act at, or upstream of, the mitochondria. Moreover, activated PKC-δ binds directly to the carboxyl-terminus of DNA-dependent protein kinase (DNA-PK), which plays an essential role in the repair of DNA double-strand breaks. Following binding, DNA-PK is phosphorylated and this results in its dissociation from DNA, the inhibition of DNA repair and enhanced DNA fragmentation (Brodie and Blumberg, 2003).

Nevertheless, yet another PKC isoform, PKC-ßII, was recently identified as an apoptotic lamin B kinase in pyF111 rat fibroblasts treated with etoposide (Chiarini et al., 2002). In this case, it was found that large complexes consisting of PKC-ßII, lamin B1, and active caspase-3 and -6 could be immunoprecipitated from the nuclear envelope of etoposide-treated, but not of control, cells. PKC-ßII moved from cytoplasm to the nuclear envelope in response to etoposide, while hispidin, a selective inhibitor of PKC-ßs, partly reduced lamin B phosphorylation and completely blocked its proteolytic degradation even in the presence of active caspase-6. In this experimental system, lamin B-bound PKC-ζ was inactive, whereas PKC-α did not combine with the lamin.

Therefore, we can infer that either different cells may have distinct PKC isozymes which act as lamin B apoptotic kinases or that different apoptosis inducers activate, in the same cell type, distinct PKC isotypes all capable of cleaving lamin B (Fig. 2).

Nevertheless, nuclear PKC isoforms might also be involved in the protection from apoptosis. In this sense a good candidate seems to be PKC-ζ (Mizukami et al., 1997; Cataldi et al., 2003). It is completely unknown how PKC-ζ could protect cells from apoptotic stimuli.

Concluding remarks and future perspectives

More than 15 years after the presence of PKC was for the first time demonstrated in rat liver nuclei (Capitani et al., 1987), a wealth of evidence has been collected about the possible involvement of this family of enzymes in the regulation of many events taking place in the nucleus. The key step to understanding the exact roles played by nuclear PKC is the identification of its substrates in this organelle and the analysis of changes in their functions caused by PKC-dependent phosphorylation. Several nuclear substrates have been identified but, surprisingly, we still lack information on the physiological significance of the phosphorylative events. Therefore, this issue needs to be further addressed. Although substantial advances have been made in the field of nuclear PKC, there seems little doubt that a large body of information is still to be collected. In particular, the functions of the different isozymes identified at the nuclear level, their substrate specificities and interacting partners, and the mechanisms that mediate their translocation to the nucleus remain vague. They should, therefore, be defined in more detail. Molecular biological approaches should now make this possible: (i) stable overexpression of PKC isozyme cDNAs under the control of constitutive and inducible promoters and (ii) antisense mRNA strategy can increase or decrease, respectively, the levels of individual isozymes leading to a better understanding of their specific functional roles; and (iii) overexpression of selectively mutated PKC cDNAs to abolish nuclear localization motifs should definitely clarify the roles played by PKC isotypes in the nucleus. These types of experiments should solve a recurring problem in the assessment of the effective role played by nuclear PKC isoforms in the regulation of cell responses. Overexpression of a given isotype almost invariably results in enhanced levels of the enzyme both in the nucleus and in the cytoplasm, as exemplified by a recent study on the importance of PKC-ζ in the induction of an erythroid phenotype in U937 leukemia cells (Mansat-De Mas et al., 2002). Indeed, it is in this case almost impossible to tell the respective contribution of the two

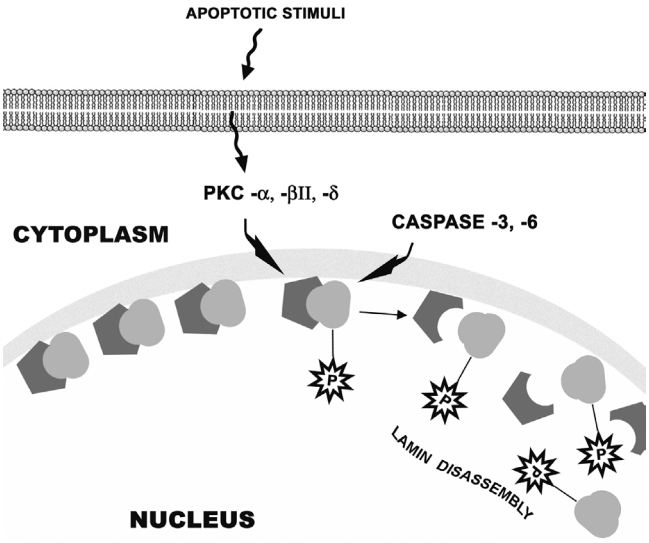

Fig. 2. Involvement of nuclear PKC isoforms in the apoptotic process. Several apoptotic stimuli induce translocation to the nucleus of different PKC isoforms. All of these isozymes seem to target lamin B which is phosphorylated on an unidentified residue. Lamin B phosphorylation facilitates its cleavage by caspase-3 or -6, which is then followed by lamin disassembly, a critical step in apoptotic nuclear collapse. Note that in the diagram we did not take into consideration cleavage of lamin A that, in some experimental models (MCF-7 cells for example, see Ruchaud et al., 2002), is performed by caspase-6 and is essential for the initiation and completion of the typical apoptotic nuclear changes. Very recent results point to the likelihood that also phosphorylation of lamin A/C is important in the context of apoptosis (Steen et al., 2003).

pools of the enzyme to cell differentiation.

Furthermore, forced expression of chimerae of the regulatory and catalytic domains of different PKC isozymes should provide the identification of the sequences required for interactions with proteins and lipids at the nuclear level, thereby allowing the designing of new classes of PKC inhibitors and activators with the potential for isotype selectivity.

Other unexpected scenarios involving PKC isoforms in the nuclear compartment are beginning to emerge, as demonstrated by cPKC translocation to the pronucleus of fertilized porcine oocytes (Fan et al., 2002).

Finally, the contribution of aberrant expression or regulation of nuclear PKC in a wide range of human diseases ranging from cataract to atherosclerosis and to cancer, must be further investigated and it is a challenging possibility for the future that nuclear PKC isotypes and their substrates will provide important targets for development of novel therapeutic agents. Indeed, in the past few years a large number of PKC activators and inhibitors with potential as anticancer agents have been developed and several of these compounds are already in Phase II clinical testing (Hofmann, 2001; Barry and Kazanietz, 2001).

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