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Regulation of protein kinase C and role in cancer biology

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Abstract

Protein kinase C (PKC) is a family of closely related lipid-dependent and diacyglycerol-activated isoenzymes known to play an important role in the signal transduction pathways involved in hormone release, mitogenesis and tumor promotion. Reversible activation of PKC by the second messengers diacylglycerol and calcium is an established model for the short term regulation of PKC in the immediate events of signal transduction. PKC can also be modulated long term by changes in the levels of activators or inhibitors for a prolonged period or by changes in the levels of functional PKC isoenzymes in the cell during development or in response to hormones and/or differentiation factors. Indeed, studies have indicated that the sustained activation or inhibition of PKC activity *in vivo* may play a critical role in regulation of long term cellular events such as proliferation, differentiation and tumorigenesis. In addition, these regulatory events are important in colon cancer, where a decrease in PKC activators and activity suggests PKC acts as an anti-oncogene, in breast cancer, where an increase in PKC activity suggests an oncogenic role for PKC, and in multidrug resistance (MDR) and metastasis where an increase in PKC activity correlates with increased resistance and metastatic potential. These studies highlight the importance and significance of regulation of PKC activity *in vivo*.

Introduction

Protein kinase C was initially discovered and characterized by Nishizuka and co-workers in 1977 as a proteolytically activated kinase [1]. PKC was then further characterized as a calcium and phospholipid (PL) dependent kinase [2]. Two major discoveries in the early 1980's established the importance of PKC in signal transduction and tumor promotion. The first was that the basal activity of PKC was stimulated by diacylglycerol (DAG), a product of the phosphatidylinositol (PI) cycle [3]. Shortly thereafter came the discovery that PKC was activated by and was the major intracellular receptor for the tumor promoting phorbol esters [4, 5]. Other work in the early 1980's clarified the mechanisms by which calcium, phospholipid and DAG activated PKC *in vitro*, and led to the characterization of many activators, inhibitors, and substrates for PKC. At the same time the phenomena of autophosphorylation, pseudosubstrate inhibition, translocation and down regulation of PKC were also elucidated and studied.

Much of the early work characterizing the enzymatic regulation of PKC assumed that PKC was a single enzymatic entity, however, cloning of PKC has revealed that PKC is a family of closely related isoenzymes; products of distinct genes (with the exception of PKC βI and βII which are derived via alternative splicing of a common gene). As of this date eleven different PKC isoenzymes have been cloned including the calcium-dependent isoenzymes, α, βI βII, and γ, the calcium-independent isoenzymes δ, ε, η, θ and μ and the atypical isoenzymes ξ and i(λ).

The PKC isoenzymes are closely related structur-

ally; composed of a single polypeptide chain divided into 2 domains: a regulatory domain at the amino terminus and a catalytic domain at the carboxyl terminus. The enzyme can be divided into 4 regions conserved across isoenzymes (C1–C4) and 5 variable regions (V1–V5) which are variable between isoenzymes but conserved within an isoenzyme across species [6].

Functions for most of the regions of PKC have been proposed. The C1 region contains the pseudosubstrate site thought to inhibit the enzyme by binding to the catalytic site [7]. The C1 region also contains the tandemly repeated cysteine rich regions which bind phorbol esters and DAG [8, 9]. PKC ξ and λ contain only one cysteine rich region, thus these isoforms are unresponsive to phorbol esters and DAG. Recently, expression of the second cysteine rich region of PKC γ as a GST fusion protein along with mutational studies of this region have defined a 43 amino acid region (residues 102–144 of this isoform) which is sufficient for phorbol ester binding [10, 11]. In addition, one cysteine rich region was sufficient for lipid dependent, stereospecific phorbol ester and DAG binding, although the lower cooperativity and affinity of this binding compared to the intact PKC γ leaves the possibility that two cysteine rich regions are necessary for efficient binding *in vivo*. The C2 region is thought to contain the calcium binding site since the calcium-independent enzymes lack this region, however, no classical calcium binding sequences such as an EF hand are present [12]. Recently mutational studies have suggested that the C2 region forms a calcium specific binding domain with the C1 region [13]. The C3 region contains an ATP binding site with three glycine residues and a downstream lysine except in PKC ξ where the last glycine is replaced by an alanine. The V3 region is the hinge region, cleaved by calpain or trypsin to separate PKC into a calcium, PL independent protein kinase and a phorbol ester receptor [12, 14]. The other variable regions V1, V2, V4, V5 are conserved within an isoenzyme across species suggesting that they have functional roles that may distinguish the isoenzymes in terms of substrates, localization, or regulation.

The various biochemical properties of PKC isoenzymes and regulation by lipid cofactors have been reviewed recently [15–18]. This review will concentrate on the role of PKC in regulating the long term cellular events, proliferation and differentiation, in both normal and transformed cells. We would like to contrast the classical mode of PKC activation (short term activation by second messengers which is critical for signal transduction and controlling short term events mediated by PKC such as secretion) with long-term regulation of PKC activity (by modulating the levels and activities of various PKC isoenzymes and PKC activators) which we believe is important for the regulation of long-term cellular events such as proliferation and differentiation. Finally, we would like to highlight the importance of PKC and the regulation of PKC activity in cancer biology by examining the role of PKC in colon and breast cancer as well as the involvement of PKC in metastasis and multidrug resistance.

Role of protein kinase C in signal transduction: transduction of mitogenic signals

Regulation by DAG

Physiologically, PKC is activated by the concerted action of PL and the second messenger, DAG. In addition, some isoenzymes (α, βI, βII and γ) are also calcium-dependent. The interactions between PKC and calcium PL, and DAG have been worked out using a mixed-micelle assay for PKC [19]. These studies suggest a two step mechanism for the activation of PKC, one in which the enzyme associates with the membrane by association with PL and calcium and the second in which it becomes activated by DAG [19, 20]. Use of the mixed micelle assay has also allowed a detailed examination of the stoichiometry and specificity of the interaction between PKC and DAG. These studies found that one molecule of DAG interacts with one molecule of PKC to cause activation. Also, the structure and stereospecificity of DAG are critical for its ability to activate PKC. Thus, even though sn-1,2-DAG is the active species, 1,3-DAG and sn-2,3-DAG are unable to activate PKC [16].

Regulation by free fatty acids

In addition to activation by calcium, PL, and DAG or phorbol esters, PKC can also be activated by cis-unsaturated fatty acids such as oleic and arachidonic acids (AA). Activation of PKC by fatty acids appears to occur independently of PS [21] while the involvement of DAG has been the subject of controversy with some studies showing that fatty acid activation is independent of DAG [22] and others showing strong synergy between fatty acids and DAG [23, 24]. Studies on the effect of calcium have also yielded conflicting results with some reporting calcium-independent fatty acid activation [21] and others reporting calcium-dependent activation [22]. Studies in this laboratory have distinguished the mechanism of activation of PKC by fatty acids and interaction of PKC with fatty acids from that of calcium, PL and DAG in several ways. First, sodium oleate appears to be unable to inhibit phorbol ester binding to PKC indicating that fatty acids interact with PKC at a site distinct from the phorbol ester/DAG binding site [24]. Second, certain PKC inhibitors such as sphingosine as well as conditions known to inhibit PKC activity such as high ionic strength are unable to inhibit oleate-induced activation of PKC to the same extent as PS/DAG-induced activation. Sodium oleate also does not induce autophosphorylation of PKC, fails to interact with membrane bound PKC, and does not cause aggregation of PKC with substrate. Finally, sodium oleate appears to activate preferentially soluble rather than membrane bound PKC [24]. Further studies have indicated that only free sodium oleate is able to activate soluble PKC [25].

Taken together, these results allowed a model for PKC activation to be formulated. As shown in Fig. 1, binding of ligands to appropriate receptors leads to activation of phospholipase C (PLC) releasing inositol triphosphate (IP_3) and DAG and/or activation of phospholipase A_2 (PLA_2), releasing arachidonic acid. IP_3 interacts with its receptor to cause the release of intracellular calcium stores and a rise in intracellular calcium [26] which, among other things, may aid in recruiting cytosolic PKC to the membrane (translocation) where it remains inactive but primed for activation. On the membrane, PKC interacts with 4 PL molecules, either via a calcium bridge or directly, and with one DAG to become a fully activated kinase now ready to phosphorylate cellular proteins [27, 28]. Alternatively, free arachidonic acid can activate PKC in the cytosol. Thus, activation of PKC during signal transduction can occur in two compartments with soluble PKC being the target for the second messenger arachidonic acid and membrane bound PKC being the target for the second messenger DAG [25]. The regulation of PKC by the second messengers DAG and arachidonic acid has firmly established PKC's role in signal transduction.

Transduction of mitogenic signals of growth factors

Many of the signals transduced by PKC are mitogenic signals sent by growth factors (e.g. PDGF and EGF). PDGF binds to its high affinity receptor (PDGFR) and activates the receptor's intrinsic tyrosine kinase activity to mediate a number of cellular effects including initiation of DNA synthesis and induction of c-fos and c-myc expression [29]. PKC has an integral role in the PDGF pathway as most of the processes initiated by PDGF treatment can be mimicked by the PKC activators PMA or synthetic diacylglycerol (AOG) and, conversely, these phenomena can be blocked by downregulation of PKC. In addition, PDGF stimulates PI turnover by activating PLC-γ resulting in increased DAG and intracellular calcium, the stimuli which activate PKC [30]. Thus, PDGF can activate PKC *in vivo* as measured by the induction of phosphorylation of the MARCKS protein, a key substrate for PKC [29]. Mutational analysis of the PDGF tyrosine kinase domain has indicated that PI turnover via PLC generating DAG and calcium is not sufficient for the mitogenic response and that another signal, perhaps via the associated PI-3 kinase activity, is necessary [31, 32]. PKC also has a role in the EGF signalling pathway. When PDGF stimulates the PKC pathway, the EGF signalling pathway is downregulated [33]. The downregulation of this pathway, which can be mimicked by PMA, is due to a decrease in the affinity of the EGF receptor for EGF without a decrease in receptor number. Further

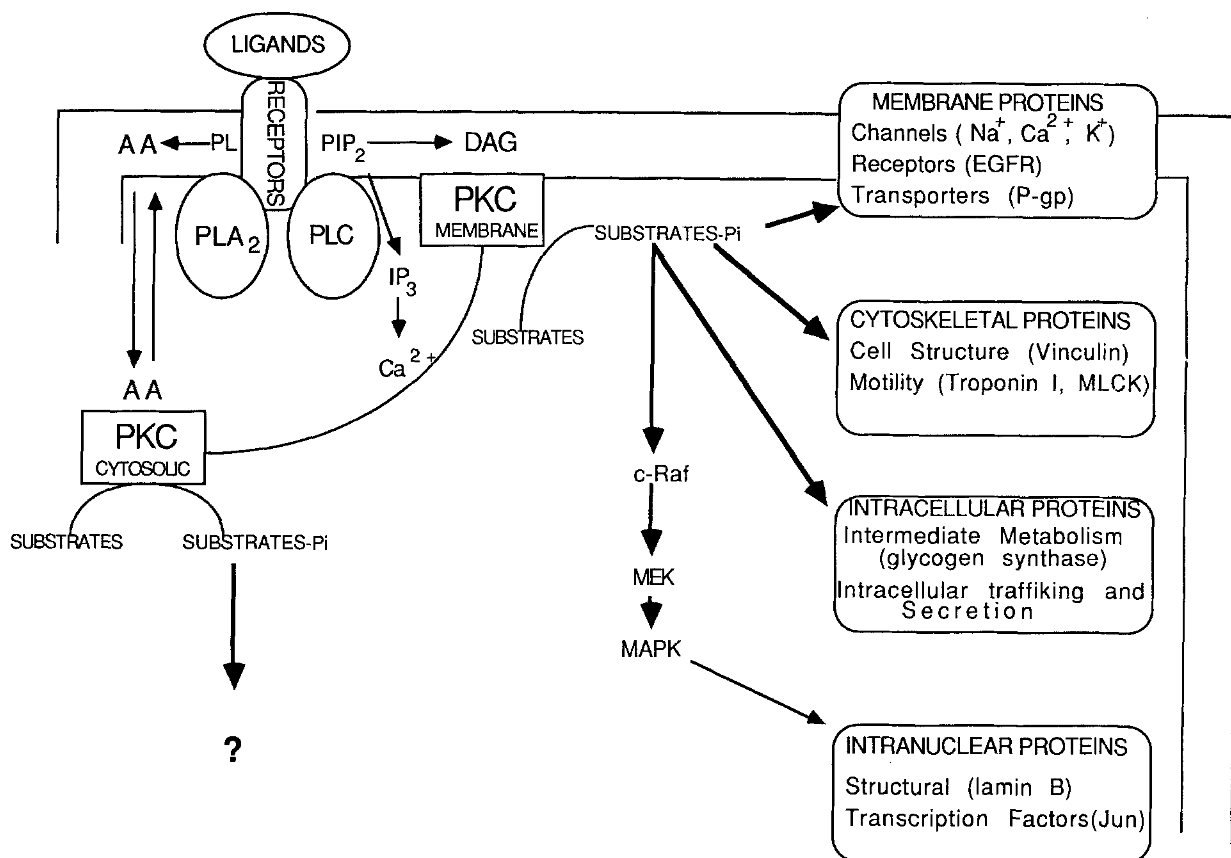

Fig. 1. Protein kinase C mediated signal transduction and biology. The interaction of ligand with its respective receptor initiates the signal transduction cascade. Coupling with PLC results in generation of DAG which activates membrane associated PKC and IP_3 which increases intracellular calcium. Coupling to PLA_2 generates arachidonic acid (AA) which can activate cytosolic PKC. Activation of PKC results in the phosphorylation of physiological substrates which mediates the subsequent biology including modulation of membrane, cytoskeletal, cytosolic and intranuclear processes. The pathway of PKC mediated signal transduction to the nucleus via Raf, MEK and MAPK has recently been elucidated.

studies have indicated that PKC directly phosphorylates the EGF receptor, suggesting that activated PKC acts directly on the EGF receptor to decrease its affinity for EGF [33]. Mutational studies in which the residue (Thr654) on the EGFR phosphorylated by PKC *in vitro* and *in vivo* is mutated to a tyrosine support this model [33]. However, mutation to alanine still allows downmodulation by PKC suggesting that other residues or another mechanism is also involved [34].

More recent studies have elucidated the molecular details in pathways for transduction of mitogenic signals and have identified the possible role(s) for PKC in this pathway. As shown in Fig. 1, the binding of PDGF and other growth factors to their respective receptors activates their tyrosine kinase activity. This either directly or indirectly (through the GRB-2/Sos/Ras pathway) activates PLC to cause PI turnover and increases in DAG and intracellular calcium activating PKC [30, 35]. PKC is then able to phosphorylate and activate c-Raf-1, a serine-threonine protein kinase [36]. c-Raf-1 then phosphorylates and activates mitogen activated protein (MAP) kinase kinase (MEK) which subsequently phosphorylates MAP kinase leading to the direct phosphorylation of transcription factors such as Jun in the nucleus and activating the mitogenic program in the nucleus [37].

Role of protein kinase C in tumor promotion: PKC as the major cellular phorbol ester receptor

Activation, translocation and downregulation of PKC

In 1982, Nishizuka and coworkers reported that PKC in association with PL was directly activated by the tumor-promoting phorbol esters [4]. In addition, other studies showed that PKC was the major intracellular receptor for phorbol esters [5]. Phorbol esters activate PKC in a manner analogous to the endogenous activator, DAG, except that phorbol ester activation can be maintained for prolonged periods of time due to their metabolic stability. Thus, prolonged stimulation of PKC has been proposed to be the mechanism for the tumor promoting action of the phorbol esters [4]. However, the action of phorbol ester on PKC results not only in activation, but also in translocation of PKC to the membrane and subsequent downregulation. This raises the possibility that tumor promotion may be a consequence of either prolonged activation or the subsequent inactivation of PKC by phorbol esters. In any case, the effect of phorbol esters on PKC was the first indication that prolonged activation or inhibition of PKC activity could play a critical role in cell regulation. Since these initial reports, PKC has also been shown to be regulated by many other tumor promoters such as byrostatins, unsaturated free fatty acids, and possibly by additional organic compounds such as benzene, chloroform and carbon tetrachloride [38–40], firming the link between PKC and tumor promotion.

Role of PKC in skin cancer/epithelial differentiation

Long before phorbol esters were known to activate PKC, their role in the promotion of skin cancer in animal models had been well characterized. The ability of a single dose of PMA to induce skin cancer in an appropriately pre-treated mouse and also to completely downregulate PKC in a long term fashion (3–4 days) implicated PKC in this process [41]. Further studies have linked PKC, not only to skin cancer, but also to the normal processes of epithelial growth and differentiation. Keratinocytes, which make up 99% of skin epithelium, undergo a defined process of growth and differentiation to continually supply fresh epithelium. At the epidermal/dermal junction, basal cells continually divide to provide a source of cells which migrate to the surface as they differentiate from spinous, to granular to cornified keratinocytes [42]. In models of this differentiation process, induction of differentiation by increasing extracellular calcium causes an increase in PI-specific PLC activity, increases in DAG and IP_3, an increase in intracellular calcium, changes in PKC activity, alteration of PKC localization, and changes in markers of differentiation such as Fos expression. These studies implicate activation of the DAG/PKC pathway in association with differentiation. In intact epidermis, PKC activity has been shown to be linked to the specific transition from spinous to granular cells [43]. Recently, PKC η has been found primarily in epithelial cells (skin and lung) and localization studies have found PKC η in the differentiating or differentiated epithelial cells but not in the basal undifferentiated layer [44]. In addition, subcellular localization studies have localized PKC η to the nucleus [45]. Thus, PKC η appears to be poised to have a role in epithelial differentiation. *In vitro* models of differentiation and transformation of keratinocytes have supported such a role. In one study in which keratinocytes were transformed with H-ras and calcium stimulation, levels of PKC η increased dramatically while levels of PKC ξ decreased [46]. In another study examining terminal differentiation of keratinocytes by PMA, again PKC η levels were increased but both PKC α and δ expression were decreased [47]. In another model, PKC has been implicated in the regulation of melanocyte growth since normal melanocytes require PMA to grow in culture while the growth of transformed melanocytes is repressed by PMA. In addition, a chronic decrease in PKC activity, due to a decrease in PKC α, δ and ε protein levels has been correlated with growth [48, 49]. Thus, in the epithelium, long-term changes in PKC activity either through the action of phorbol esters or by specific changes in PKC isoenzyme levels leads to either growth in melanocytes, differentiation of keratinocytes or transformation.

Role of protein kinase C in differentiation and proliferation: long term regulation of PKC isoenzyme levels

Overexpression of PKC isoenzymes

The initial studies on the ability of phorbol esters to cause tumors along with the cloning of PKC isoenzymes led to studies examining the effects of overexpressing normal and mutant PKC isoenzymes *in vitro*. The first and most widely studied overexpression was that of PKC βI in a number of cell lines. Initially, PKC βI was overexpressed in rat fibroblasts where, along with a 20–50 fold increase in PKC activity, the cells were partially transformed as these cell grew to a higher saturation density, were more anchorage-independent and were able to form tumors in nude mice [50]. Shortly thereafter, PKC γ was overexpressed in NIH 3T3 cells resulting in cells with reduced growth factor requirements, growth to higher saturation density and formation of tumors in nude mice [51]. Normal and mutant PKC α was also overexpressed in fibroblasts with one study finding that mutant PKC α was transforming and a latter study disputing these results [52, 53]. Subsequent studies with PKC βI showed that while PKC βI is able to act as an oncogene in some cells (fibroblasts) it can have the opposite effects in other cellular contexts. Thus, when PKC βI was overexpressed in the colon cancer cell line HT29, the cells doubling time increased, they grew to a lower saturation density, had decreased anchorage-dependent growth *in vitro*, and displayed reduced tumorogenecity in nude mice [54]. Similar results have been shown with overexpression of PKC δ in NIH-3T3 cells [55] while the overexpression in PKC ε in NIH-3T3 cells and Rat 6 fibroblast cell lines was transforming as measured by decreased anchorage-independence and increased tumor formation in nude mice [55, 56]. Taken together these studies extend the observation that long-term changes in the levels of PKC isoenzymes and PKC activity could have profound effects on cellular proliferation and differentiation.

Tissue-specific and developmentally regulated expression of PKC isoenzymes

The development of isoenzyme-specific antibodies and isoenzyme-specific nucleotide probes has allowed for the extensive investigation of the distribution and regulation of expression of PKC isoenzymes by Western blot analysis, Northern blot analysis, *in situ* immunocytochemistry and *in situ* hybridization. Many observations have now confirmed that PKC isoenzymes are differentially expressed and that this expression can be regulated. The initial studies on the tissue expression of distinct PKC isoenzymes revealed a highly variable distribution as summarized in Table 1. While some tissues such as brain contain all isoenzymes, others such as skin and skeletal muscle contain only a few. At the same time some isoenzymes such as α and ξ are ubiquitously expressed while others such as γ and η are expressed in only a few tissues. The cellular localization of specific isoenzymes is also distinct. For example although α, βI, βII, γ, δ and ξ are all expressed in the cerebellum, α, γ, δ, ε, and ξ are in Purkinje cells while βI is in the granular layer and βII is in the molecular layer of the cerebellar cortex [57]. Studies on the levels of PKC isoenzymes during development revealed that the PKC genes are also expressed in a developmentally regulated fashion as summarized in Table 2. For example, with the PKC β gene products PKC βI and βII, low levels are observed in the fetal spleen and during the first 2 weeks of life after which a rapid increase in expression occurs. However, in the thymus, maximal levels occur shortly after birth with a decrease in expression thereafter. These levels correspond to the developmental patterns of these two organs suggesting that PKC βI and/or βII play an important role in their development [58]. Interestingly, the decrease in expression of PKC β in the thymus correlates with the involution of the thymus by regulated cell death or apoptosis. Since activated PKC has been demonstrated to protect against apoptosis and downregulation of PKC can enhance cell death [59], the decrease in PKC β expression provides an attractive mechanism for a decrease in PKC activity at the tissue level allowing involution and negative selection in the thymus to occur.

Table 1. Tissue and cell specific expression of protein kinase C isoenzymes

	PKC α	PKC βI	PKC βII	PKC γ	PKC δ	PKC ε	PKC η	PKC ξ	PKC θ	PKC i (λ)
Tissue										
Brain	+	+	+	+	+	+	+	+	+	+
Liver	+	+	+	−	+	+		+	+	+
Spleen	+	+	+	−	+	+	+			+
Heart	+	+	+	−	+	+	+	+	+	+
Lung	+	+	+	−	+	+	+	+	+	+
Kidney	+	+	+	−	+	+		+		+
Pancreas	+	+	+			+				+
Skin				−	+		+	+		+
Adrenal gland	+	+	+	+	+	+		+		
Pineal gland	+			−	+	+		+		
Pituitary gland		+	+	−				+		
Ovary	+	+	+	−	+	+		+		+
Testis	+	+	+	−	+	+		+		+
Smooth muscle	+	+	+			+		+		
Skeletal muscle									+	−
Retina	+	+	+		+	+		+		
Uterus					+					+
Intestine					+					+
Placenta					+				+	
Thymus	+	+	+	−		−				
Primary cells										
B-lymphocytes	+	+	+	−	+	+	+	+		
T-lymphocytes	+	+	+	−	+	+	+	+		
Platelets	+	+	+	−	+	+		+	+	
Neutrophils	+	+	+	−						
Fibroblasts	+	−	−	−	+	+		+		

(+) indicates expression of the PKC isoenzyme in that particular tissue either at the mRNA or protein level as detected by Northern blot, Western blot, or *in situ* histochemical analysis. (−) data is only indicated where Northern blot analysis has failed to detect expression. Data is compiled from the following references ([137], [138], [139], [58], [140], [141], [142], [143], [144], [145], [146], [147], [148], [149], [150], [151], [152] and [153]). For expression in cell lines see [18].

Regulated expression of PKC isoenzymes

The distinct cellular expression of PKC isoenzymes, their developmental regulation, and ability to alter cell regulation when overexpressed all suggest that these isoenzymes play distinct roles *in vivo*. Moreover, control of the respective levels of PKC isoenzymes may be critical for proper function. To examine this, the expression of PKC isoenzymes at both the protein and RNA level in response to various cellular stimuli have been examined. The results of these studies are summarized in Table 3. Two major themes arise from these studies. First, the levels of distinct PKC isoenzymes can be dramatically changed (either increased or decreased) in response to a wide variety of inducers and in a variety of situations. Studies have found changes in expression of every PKC isoenzyme which has been biochemically and immunologically characterized (α–η) and the inducers vary from differentiation inducing agents such as retinoic acid and Vitamin D3 to iron/transferrin, ethanol and transfection with H-ras. The second finding is that the mechanisms regulating the levels of specific PKC isoenzymes span the entire gammut of potential regulatory mechanisms, from transcriptional regulation of the individual PKC genes, to regulation of the post-transcriptional processes, mRNA splicing and mRNA stability as well as translational and post-translational controls.

Table 2. Developmental regulation of protein kinase C isoenzyme expression

Organ, tissue, or cell (species)	PCK isoenzyme(s)	Changes in mRNA expression	Reference
Brain	α	low at birth, increase after birth until 3 weeks	[58]
(rat)	β	low at birth, increase after birth until 3 weeks	
	γ	low until 1 week after birth, then increase until 2–3 weeks	
Brain	α	low until 6 weeks after birth, then increase to 9 weeks (10 fold)	[154]
(human)	βI	low to absent until 6–9 weeks after birth then increase to adulthood (30 fold)	
	βII	low until 6–9 weeks after birth then increase to adulthood (30 fold)	
	γ	low expression until increase in adulthood	
Cerebellum	α	low at birth, increase after birth until 3 weeks	[155]
(rat)	βII	high at birth, then decreases	
	γ	low at birth, increase until 2–3 weeks, then decrease	
Spleen (rat)	β	low until 2 weeks after birth, then increases	[58]
Spleen	α	low until 3 weeks after birth, then increase	[142]
(mouse)	β	low until 3 weeks after birth, then increase	
Thymus (rat)	β	high at birth, decrease thereafter	[58]
Thymus	α	high at birth, decrease thereafter	[142]
(mouse)	β	high at birth, decrease thereafter	
	ξ	high at birth, decrease thereafter	
B-lymphocytes	α	increases from pre-B cell to plasma cell	[144]
(mouse)	β	decreases from pre-B cell to plasma call	
Thalamus (mouse)	δ	absent in embryo, neonate, increases at 1–2 weeks until adulthood	[156]

Role of regulation of PKC isoenzyme levels in differentiation and proliferation

These studies have been particularly insightful into the mechanism of regulation of proliferation and differentiation by PKC isoenzymes. One important model for the study of cellular differentiation and proliferation is the human promyelocytic leukemia cell line HL-60. HL-60 cells are largely undifferentiated cells that maintain the ability to undergo several different pathways of differentiation depending on the inducer with which they are stimulated [60]. For example, treatment with 1,25-dihydroxy vitamin D_3 results in differentiation along the monocytic pathway while treatment with PMA results in a macrophage-like phenotype. Besides the capability of phorbol esters to stimulate differentiation in this cell line, several lines of evidence implicated PKC in the differentiation o. HL-60 cells. First, inhibitors of PKC such as sphingosine were able to block PMA induced differentiation [61]. Second, continual exposure of HL-60 cells to diacylglycerol was able to mimic PMA induce differentiation [62]. Finally, Vitamin D_3-induced differentiation of HL-60 resulted in an increase in PKC protein as measured by an increase in phorbol ester receptors [63]. While investigating the mechanism of the increase in PKC protein levels we determined that transcriptional activation of the PKC α and PKC β genes resulted in an increase in steady state levels of PKC α and PKC β mRNA and thus increases in PKC α and PKC β protein [64]. The increase in PKC protein resulted in an increase in PKC activity as measured by the increase in phos-

phorylation of several PKC substrates *in vivo*. This long term increase in PKC activity occurred in the absence of changes in DAG levels. Thus, we proposed that while PKC activity may be increased in the short term by increasing DAG levels, more long-term regulation of PKC activity in the cell could be achieved by regulating the levels of PKC isoenzymes. In order to define the role of specific PKC isoenzymes in Vitamin D3 induced differentiation of HL-60 cells, we have used antisense technology to selectively decrease PKC α or β (βI and βII) protein levels [65]. We have found that antisense to PKC β can abrogate the increase in PKC βI and βII expression and in turn inhibit Vitamin D_3-induced differentiation (up to 50%) without affecting proliferation. Importantly, although antisense to PKC α could decrease PKC α protein levels, this treatment had no effect on Vitamin D_3 induced differentiation. Thus we have demonstrated that the increase in PKC β gene expression leading to an increase in both PKC βI and βII protein levels is necessary for full induction of differentiation. At the same time we have been able to dissociate the effects of PKC β on differentiation and proliferation in this cell line.

Long-term alterations in PKC activity by alteration of levels of PKC activators and inhibitors

Another mechanism for changing PKC activity on a long term basis, is to modulate the production of endogenous activators of PKC such as DAG and free fatty acids. Indeed, one effect of ras transformation is a large increase in DAG production which could activate PKC for prolonged periods of time [66]. On the other hand, long term increases in DAG production could also mimic the action of long-term phorbol ester treatment by causing downregulation of PKC as has been shown in melanoma cells [49]. The production of other activators of PKC, such as free fatty acids and lysoPC via the PLA_2 mediated breakdown of PC and longer term generation of DAG via the PLD mediated breakdown of PC may also play an important role in long term alteration of PKC activity *in vivo* [67].

The activity of PKC is also known to be modulated *in vivo* by endogenous inhibitors. The presence of these endogenous inhibitors was first inferred from the observation that partial purification of PKC is required for accurate measurement of PKC activity *in vitro*. Indeed, several endogenous inhibitors have been described and a few have been cloned and characterized. The best understood are the proteins in the 14-3-3 family of proteins which are ubiquitously expressed and potently and specifically inhibit PKC activity *in vitro* [68, 69]. Despite these studies, the investigation of endogenous inhibitors of PKC has lagged behind those of activators and thus, this area requires further investigation.

PKC involvement in human cancers

Besides being linked to the processes of tumor promotion, transformation and regulation of cellular differentiation and proliferation, PKC has been directly linked to the pathogenesis of several human cancers including skin cancer as discussed above as well as colon cancer and breast cancer.

Role of PKC in colon cancer

Colon cancer, along with lung cancer and breast cancer, is one of the most common human malignancies and the second leading cause of cancer death in the United States. The risk factors for colon cancer include an increased consumption of dietary fat, decreased consumption of fiber and a history of colonic inflammatory disease or polyposis. Oncogenes such as ras, as well as the tumor suppressor genes p53, fap (familial adenomatous polyposis) and DCC (deleted in colon cancer) are also thought to be involved [70]. Recently, mutations in a DNA mismatch repair gene homologous to the mutS gene, have been linked to Hereditary Nonpolyposis Colorectal Cancer, suggesting that antioncogenes may be involved here as well [71, 72]. Several experimental findings have also indicated that PKC is directly involved in colon carcinogenesis. First, unsaturated free fatty acids and bile acids, both of which are present in the colon, can act as tumor pro-

Table 3. Regulation of protein kinase C isoenzyme expression

Cells, cell line, or tissue (species, tissue)	Inducer(s), variable and/or (Effect)	PKC Isoenzymes	Changes in expression	Level/regulation	Reference
SHE fibroblasts (syrian hamster, embryo)	ionizing radiation (transformation)	β	increase (4-6 fold)	mRNA/transcriptional	[185]
R6 fibroblasts (rat, embryo)	H-ras or v-src transfection (transformation)	α	increase (4–5 fold)	mRNA/transcriptional	[157, 158]
		δ	increase (several fold)		
		ε	decrease (6–10 fold)		
HL-60 (human, leukemia)	Vitamin D3 (differentiation)	α	increase (12 fold)	mRNA/transcriptional	[64]
		β	increase (7 fold)		
CCRF-CEM (human, T-lymphoblastoid)	iron/transferrin (proliferation)	β	increase (3-fold)	mRNA/transcriptional	[159]
HL-60-PE (human, leukemia)	long term PMA (Phorbol Ester Resistance)	βI, βII	increase (4–5 fold)	mRNA/transcriptional message stability	[160]
B16 (mouse, melanoma)	Retinoic Acid (differentiation)	α	increase (10–12 fold)	mRNA/transcriptional post-transcriptional	[161, 162]
BJA-B to IM-9 (human, B-lymphoblastoid)	(differentiation?)	βI	decrease (3 fold)	mRNA/alternative splicing	[163]
		βII	increase (3 fold)		
Neura 2A (mouse, neuroblastoma)	8-Bromo cAMP (differentiation)	α	decrease	mRNA/?	[142]
		ε	decrease		
LAN-5 (human, neuroblastoma)	IFN-γ or RA (differentiation)	ε	increase (several fold)	mRNA/?	[164]
K22 epithelial (rat, liver)	H-ras transfection & (transformation)	α	increase (10–20 fold)	mRNA/?	[157]
HL-525-PE (human, leukemia)	long term PMA (Phorbol Ester Resistance)	β	decrease (4–5 fold)	mRNA/?	[165]
Thymocytes (mouse, fresh)	Concanavalin A, PMA	α	decrease (several fold)	mRNA/?	[166]
	"	β	decrease (several fold)		
	"	δ	increase (several fold)		
	Concanavalin A	ε	decrease (several fold)		
	Concanavalin A, PMA (activation)	ξ	decrease (several fold)		
EL-4 PE (mouse, thymoma)	long term PMA (Phorbol Ester Resistance)	ε	decrease (several fold)	mRNA/?	[186]
CA cell line (human, B-lymphoblastoid)	anti-HLA Class II Ab (activation)	α	increase (several fold)	mRNA/?	[167]
		β	increase (5-fold)		
Xenopus embryo	(development)	α	increase (several fold)	mRNA/?	[168]
		β	increase (several fold)		
	Injection of PKC β mRNA (neural induction)	β	decrease (several fold)	mRNA/?	
Y1 (mouse, adrenocortical)	overexpression of apolipoprotein E (inhibition of steroidogenesis)	α	increase (several fold)	mRNA/?	[169]

Table 3. Continued

Cells, cell line, or tissue (species, tissue)	Inducer(s), variable and/or (Effect)	PKC Isoenzymes	Changes in expression	Level/regulation	Reference
MCF-7 (human, breast cancer)	long term doxorubicin (induction of MDR)	α	increase (30 fold)	mRNA/?	[116]
Daudi (human, B-lymphoblatstoid)	IFN-α	ε	increase (6 fold)	mRNA/?	[170]
P-MM-4 (human, melanoma)	(differentiation)	β	increase (several fold)	mRNA/?	[171]
U937 (human, monoblastoid)	PMA (long term)	α β ε	decrease (2 fold) decrease (2–3 fold) decrease (2–3 fold)	mRNA/?	[172]
NCI H209 (human, small cell lung cancer)	c-myc transfection (transformation)	β	increase (5–10 fold)	mRNA/?	[173]
Rat Renal Mesangial	PMA (long term)	α ε	decreased faster decreased slower	protein/stability	[174]
	removal of PMA	α ε	slower increase faster increase	protein/translational	[174]
SaOS-2 (human, osteosarcoma)	PMA (long term)	α β γ	decrease (several fold) decrease (several fold) decrease (several fold)	protein/stability and translational	[175]
MCF-7, MDA-MB-231 (human, breast cancer)	PMA	α	increase	protein/post-translational modification	[176]
Jurkat-PE (human, T-cell)	long term PMA (Phorbol Ester Resistance)	α	decrease (several fold)	protein/?	[177]
HL-60 (human, leukemia)	DMSO, RA (differentiation)	α β ε	increase (5–7 fold) increase (5-fold) increase(2-fold)	protein/?	[178]
PC-12 (human, pheochromocytoma)	ethanol (upregulation of Ca channels)	δ ε	increase (50%) increase (50%)	protein/?	[179]
	NGF (differentiation)	α βII	decrease increase	protein/? protein/?	[180] [181]
lymphocyte activated killer (mouse)	Retinoic Acid (activation)	α	increase (4 fold)	protein/?	[182]
K562 (human, erythroleukemia)	PMA (differentiation)	α βII ε ξ	increase decrease increase increase	protein/?	[183, 184]
	Sodium Butyrate (differentiation)	α βII	increase increase	protein/?	
FELC (human, erythroleukemia)	hexamethylene bisacetamide (differentiation)	βII	increase	protein/?	[184]

moters and activate PKC [73]. Bile acids can also activate PKC indirectly as they stimulate PLC activity and increase DAG production [74] while increases in calcium can block the tumor promoting effects of bile acids and fatty acids by sequestering them so that they can no longer activate PKC [75]. Second, levels of PKC activity as well as DAG levels are decreased in colon cancer relative to normal colon tissue [76, 77]. Third, the expression of the PKC β gene is decreased in colon cancer relative to normal tissue [78] and finally, overexpression of PKC βI in the colon cancer cell line HT-29, is able to act as a tumor suppressor as growth is inhibited and tumorogenicity is decreased [54]. These data suggest that in colon cancer, PKC acts as a tumor suppressor, and by decreasing the levels of PKC activity, transformation can occur. Thus, long term stimulation of PKC at the protein level by the presence of increased amounts of the tumor promoting fatty acids and bile acids may initially activate PKC but then cause long-term downregulation of the enzyme. Another mechanism for decreasing PKC activity would be decreasing levels of the endogenous activator DAG, as has been shown to occur in colon cancer tissue. Finally, PKC activity levels could also be decreased by decreasing the expression of the PKC genes as has been shown for PKC β.

Role of PKC in breast cancer

Breast cancer is the most common malignancy in women, accounting for 32% of cancer, however, very little is known about the oncogenic process involved. Risk factors include a family history of breast cancer, a history of benign proliferative disease, ingestion of dietary fat and length of exposure to unopposed estrogen stimulus [79]. Many oncogenes are overexpressed in breast cancer including c-myc, Ha-ras, erb 13 and HER-2/neu. In addition, PKC is also overexpressed in breast cancer [80]. The level of expression of PKC is also altered depending of the estrogen receptor (ER) status of the breast cancer [81]. ER positive breast cancer carries a better prognosis as they tend to be less undifferentiated, respond to hormonal therapy and tend to metastasize and recur less frequently. This subgroup of breast cancer contains lower amounts of PKC than ER negative breast cancer. Thus, a trend of increasing PKC activity as the breast cancer becomes more undifferentiated is apparent. PKC has also been associated with breast cancer through *in vitro* studies investigating the proliferative status of breast cancer cell lines and the mechanism of activation of tamoxifen, the potent anti-estrogen agent used to treat breast cancer. These studies have found that, although tamoxifen can inhibit the proliferation of breast cancer cells, not all of its actions can be explained by its blockade of estrogen receptors [82]. The finding that tamoxifen could directly inhibit PKC and that phorbol esters as well as DAG could inhibit proliferation and cause differentiation of breast cancer cells *in vitro*, suggested that PKC may contribute to the action of tamoxifen [83–86]. This argument is strengthened by the correlation between the potency of phorbol esters in inhibiting proliferation and their tumor-promoting and PKC-activating abilities. In addition, removal of phorbol esters caused a resumption in growth. Thus, for breast cancer, as opposed to colon cancer, an increase in PKC activity appears to correlate with enhanced oncogenicity and inhibiting that activity by long term PMA treatment or treatment with PKC inhibitors can decrease proliferation and oncogenicity. The mechanism for increasing PKC activity has not been established. However, most likely this will involve an increase in the expression of one or more of the PKC genes. These studies remain to be performed.

Role of PKC in multidrug resistance

Multi-Drug-Resistance (MDR) is a phenotype expressed by some tumor cell populations upon exposure to cytotoxic drugs providing cells with resistance against not only the cytotoxic agent to which they were exposed but cross-resistance against other structurally and mechanistically diverse cytotoxic natural products such as anthracyclines and Vinca alkaloids [87]. MDR has been associated with many changes in tumor cells including increased glutathione peroxidase activity, decreased levels and mutations in DNA topisomerases, decreased

levels of cytochrome P450 enzymes, overexpression of the anionic isozyme of glutathione S-transferase, altered cell membrane lipid composition, and changes in the expression and activity of PKC isoenzymes [87–89). In addition to these changes, however, MDR is most closely associated with a decrease in intracellular drug accumulation and the overexpression of a 170 kDa glycoprotein, P-glycoprotein. P-glycoprotein expression correlates with decreased intracellular drug accumulation and with the degree of drug resistance [90]. P-glycoprotein is highly homologous to bacterial transport proteins, is normally expressed at high levels in specialized epithelial cells with secretory or excretory functions [91, 92], is able to bind directly to various drugs [93] and contains an ATPase activity [94]. These studies have suggested that P-glycoprotein functions in MDR to pump the cytotoxic drugs out of the cell. Indeed, transfecting cells with the mdrl gene which encodes P-glycoprotein is sufficient to induce the MDR phenotype [95].

The MDR phenotype is also associated with changes in PKC activity and isoenzyme content and many lines of evidence implicate PKC in the regulation of this phenotype. First, drug resistant lines have altered levels of PKC and its activators including higher calcium content [96] and either higher [97–99] or lower [100, 101] PKC content than their parental lines. MDR cell lines also contain more PKC in the membrane fraction than parental cell lines suggesting intrinsic activation of PKC [98]. Second, activators of PKC are able to induce the MDR phenotype and enhance the phenotype of cells already expressing MDR. In some studies this is associated with increased phosphorylation of P-glycoprotein. These activators have included phorbol esters [97], deoxycholate [102], and OAG [103]. Third, inhibitors of PKC such as staurosporine [104], H-7 [105], calphostin C [106], calcium channel blockers, phenothiazines, antiarrythmics [107], antiestrogens [85] and synthetic peptide inhibitors [108] are able to partially reverse MDR and inhibit P-glycoprotein phosphorylation. Fourth, PKC is able to phosphorylate P-glycoprotein *in vitro* on sites similar to the *in vivo* sites [109]. Fifth, overexpression of PKC α but not PKC γ in cells expressing P-glycoprotein is able to enhance the MDR phenotype of those cells and the overexpression of PKC βI is also able to induce MDR by a P-glycoprotein independent manner [110–112). Finally, reducing the expression of PKC α by antisense can attenuate the MDR phenotype [113]. Expression of PKC can also have a role in non-P-glycoprotein mediated MDR as PKC can phosphorylate and influence the activity of topoisomerase II [114] and glutathione-S-transferase [115], both of which are altered in association with the MDR phenotype.

To investigate the mechanism of altered PKC activity in MDR, studies on the expression of PKC isoenzymes in MDR cells have been reported, however, very few have analyzed both calcium-dependent and calcium-independent isoenzymes. The most comprehensive analysis of PKC isoenzymes has been done in the MCF-7 cell line where the MDR phenotype is associated with a 30 fold increase in PKC α expression at the mRNA and protein level, and a 10 fold increase in calcium-dependent PKC activity, but a decrease in PKC ε and δ protein levels with a 10 fold decrease in calcium independent PKC activity [116]. The increase in PKC α has been localized to the nucleus in this cell line [117]. In HL-60 cells, adriamycin resistance was associated with an equal amount of PKCα and a lower amount of PKCβ than the parental line along with the induction of PKCγ [118]. An increase in the expression of PKC α and β in the P388/ADR cell line was noted [119]. Others using only antibodies and probe to PKC α, found that while MDR cell lines of both the human epidermoid carcinoma cell line KB and the murine sarcoma cell line S180 contained increased amounts of the PKC α protein, only the human KB-MDR cells overexpresssed commensurate amounts of PKC α mRNA. Thus, the mechanisms by which PKC was overexpressed in these two cell lines differed [104].

In general, PKC α tends to be overexpressed in association with the MDR phenotype. Thus, MDR is associated with an increase in the expression of PKC α in the following cell lines: the murine UV-2237 cell line [102], the KB cell line [104], the murine 180 cell line [104], the murine P388 cell line [119] and in the MCF-7 cell line [116]. In addition, the overexpression of PKC α, but not PKC γ in an MCF-7 cell line already overexpressing P-glycopro-

tein increases the MDR phenotype, and reducing the expression of PKC α with antisense can attenuate the MDR phenotype. These studies define a specific role for PKC α in modulating the MDR phenotype.

Several hypothesis have been put forward as to the mechanism by which PKC is altered in MDR cells and how this altered expression regulates MDR. One of the early hypotheses for altered PKC expression was that drugs which induce MDR inhibit PKC and this chronic inhibition leads to upregulation of PKC [120]. These authors presented evidence that chemotherapeutic agents such as doxorubicin could inhibit PKC, however, only at doses not achieved therapeutically [120]. Recent studies showing changes in the expression of specific isoenzymes (usually an increase in PKC α) explain the changes in levels of PKC activity seen in MDR cell lines [104, 116, 118]. Finally, there is one report that a reduced rate of PKC degradation may be responsible for increases in PKC activity seen in MDR cell lines [121]. Once PKC levels are altered, it may be able to phosphorylate P-glycoprotein *in vivo* altering its pump function to modulate drug efflux or it may act in other ways to impair drug influx [122]. Indeed, P-glycoprotein is phosphorylated *in vivo* on serine residues [123], this phosphorylation is enhanced by the PKC activators PMA or OAG [123] and this phosphorylation changes with the MDR status of the cell [106, 124]. P-glycoprotein is also phosphorylated *in vitro* by PKC [109] as well as by PKA [125] and this phosphorylation can modulate the function of the protein [106, 126].

Role of PKC in metastasis

Metastasis is a multistep process which allows tumor cells to escape the primary tumor mass, invade the extracellular matrix, penetrate through blood vessel walls, aggregate in the blood stream, attach to the vascular endothelium and invade into the secondary site [127]. All of these properties can be regulated with calcium levels and appear to involve the adhesive properties of the cancer cell [128]. Since PMA can cause an increase in metastases in cell lines and animal models, PKC has also been implicated in the process of metastasis [129–131]. A positive correlation between PKC activity and the ability of tumor cells to form metastases as well as the ability of inhibitors of PKC or downregulation of PKC to inhibit metastasis has strengthened this association [130, 132, 133]. The mechanism of PKC action is thought to be by modulation of cellular adhesion to the extracellular matrix in response to PKC. Many cell adhesion receptors are PKC substrates (integrins, LFA-1, ICAM-1) [128]. In addition, PKC may be involved in inducing the expression of adhesion proteins (ICAM-1) [134]. Indeed, tumor cell adhesion to endothelial cells is enhanced by phorbol ester treatment while the PKC inhibitors sphingosine, staurosporine and H-7 are able to decrease adhesion [135]. An interesting model for PKC's role in mediating tumor cell adhesion is the rat carcinosarcoma cell line W-256, where the endogenous PKC activator, 12-(S)-hydroxyeicosatetraenoic acid (12-(S)-HETE) is able to activate PKC and mimic the effects of PMA on enhancing tumor cell adhesion to the endothelium, while these effects can be blocked by inhibiting PKC activity by with H-7, calphostin, PKC down regulation or with the endogenous inhibitor of 12-HETE activity, 13-(S)-hydroxyoctadecadienoic acid (13-(S)-HODE) [128, 136].

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