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Critical Review

Contribution of Individual PKC Isoforms to Breast Cancer Progression

Alejandro J. Urtreger[1], Marcelo G. Kazanietz[2] and Elisa D. Bal de Kier Joffé[1]
[1]*Research Area, Institute of Oncology "Angel H. Roffo," University of Buenos Aires, Buenos Aires, Argentina*
[2]*Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA*

Summary

The protein kinase C (PKC) family of serine/threonine kinases has been intensively studied in cancer since their discovery as major receptors for the tumor-promoting phorbol esters. The contribution of each individual PKC isozyme to malignant transformation is only partially understood, but it is clear that each PKC plays different role in cancer progression. PKC deregulation is a common phenomenon observed in breast cancer, and PKC expression and localization are usually dynamically regulated during mammary gland differentiation and involution. In fact, the overexpression of several PKCs has been reported in malignant human breast tissue and breast cancer cell lines. In this review, we summarize the knowledge available on the specific roles of PKC isoforms in the development, progression, and metastatic dissemination of mammary cancer. We also discuss the role of PKC isoforms as therapeutic targets, and their potential as markers for prognosis or treatment response.

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Keywords Protein kinase C; breast cancer; tumor progression; prognostic marker.

THE PROTEIN KINASE C FAMILY

Protein kinase C (PKC) was originally identified as a phospholipid- and calcium-dependent protein kinase (*1*). PKC influences diverse cell functions through phosphorylation of target proteins. These cell functions involve a wide variety of fundamental physiological processes including signal transduction, modulation of gene expression, proliferation, apoptosis, and differentiation (*2, 3*). Because of their importance in cell signaling, it is logical to assume that altered levels of expression or activity of these kinases may contribute to disease, including cancer. Moreover, PKCs became even more attractive for oncology researchers when early observations showed that several PKC isozymes were activated by phorbol esters, well-known tumor-promoting agents (*4*).

Individual PKC isozymes exhibit different tissue distribution, subcellular localization, and biochemical properties, an indication that each member of the family plays specialized roles (*5*), which ultimately translates into unique relationships with disease. The current classification of PKC isoforms is based on structural and regulatory characteristics (*6*). PKC comprises 10 phospholipid-dependent serine-threonine kinases grouped into three subclasses: the "classical" (PKC α, βI, βII, and γ), which can be stimulated by Ca^{2+} and diacylglycerol (DAG) or phorbol esters; the "novel" (PKC δ, ε, η, and θ), which can be activated by diacylglycerol or phorbol esters but are Ca^{2+} independent; the "atypical" (PKC ζ and λ/ι), which are unresponsive to Ca^{2+}, diacylglycerol, and phorbol esters. The structure of classical PKCs includes four conserved domains (referred as C1–C4) interrupted by five variable regions (V1–V5). The C1 region contains cysteine-rich zinc-finger-like motifs responsible for phosphatidylserine, DAG, and phorbol esters binding. An autoinhibitory pseudosubstrate (Ps) sequence is located at the N-terminal region of PKCs that is involved in autoinhibition. The C2 region in classical PKCs is rich in acidic residues and binds Ca^{2+}. The C3 and C4 regions form the ATP- and substrate-binding lobes. Novel PKCs have an altered C2 region unable to bind Ca^{2+}, and atypical PKCs are insensitive to Ca^{2+} and have only one cysteine-rich zinc-finger-like motif that is unable to bind DAG or phorbol ester. The differences in structure and cofactor dependency between PKC isozymes are summarized in Fig. 1. The Ps motif is located at the N terminus of PKCs and closely resembles a substrate phosphorylation motif. In the absence of stimuli, the Ps motif maintains the enzyme in an inactive state by sterically blocking the catalytic domain. Cofac-

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Address correspondence to: Alejandro J. Urtreger or Elisa D. Bal de Kier Joffé, Research Area, Institute of Oncology "Angel H. Roffo," Av. San Martín 5481, (C1417DTB) Buenos Aires, Argentina.
Tel.: +5411 4504-7884. Fax: +5411 4580-2811.
E-mail: urtreger@fmed.uba.ar or elisabal@fmed.uba.ar.

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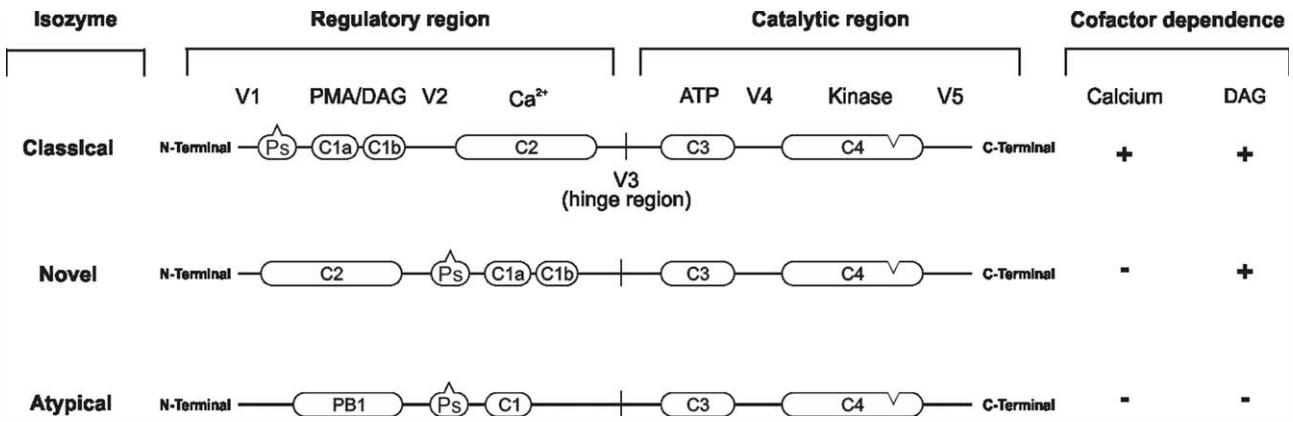

Figure 1. Structure of PKC isoforms. PKCs contain 4 conserved (C1-C4) and 5 variable regions (V1-V5). The regulatory and catalytic domains are connected through the V3 (hinge region). Novel PKCs have a C2 like domain unable to bind Ca^{2+}. The C1 domain of atypical PKCs does not have all conserved residues required for DAG binding. All PKC isoforms possess the pseudosubstrate sequence (PS) involved in maintenance of the inactive state of the molecule.

tor binding results in a conformational change that releases the Ps motif and increases the catalytic activity of the enzyme (Fig. 2).

PKC AND CANCER

There are only limited numbers of cases in which mutations in PKC isoforms are linked to a transformed phenotype. Point mutations in PKCα were found in human pituitary adenomas, follicular neoplasms, and thyroid cancers (*7, 8*). Moreover, rearrangement of PKCε was reported in a thyroid follicular carcinoma cell line, suggesting a role in tumorigenesis (*8*). On the other hand, changes in the expression levels or activation status of PKC isozymes have been reported in numerous human cancers (*9*), and in many instances, a correlation between elevated PKC protein levels and aggressiveness has been reported (*10*). Moreover, PKCs have been studied as targets for the treatment of cancer for many years. The benefits of using PKC inhibitors to control cancer have been discussed elsewhere (*11, 12*).

Based on the current knowledge, it is clear that PKC isozymes have distinct roles depending on the cell type. Intriguingly, PKC isozymes that mediate proliferative responses in some cell lines could behave as growth inhibitory in others. For example, PKCβ mediates proliferative responses in lung cancer cells and behaves as growth inhibitory in colon cancer cell lines. Similar features were described for PKCδ. In glial, vascular smooth muscle, and endothelial cells, PKCδ impinges negatively on both G1/S and G2/M cell cycle transitions upregulating the cell cycle inhibitor p21 (*13*). Moreover, PKCδ activation promotes apoptosis of colon and prostate tumor-derived cell lines by inducing cytochrome *c* release and activating caspase-3 (*14*). In addition, it has been proposed that in prostate cancer cells PKCδ can also trigger an autocrine apoptotic loop through the secretion of TNFα and TRAIL (*6*). On the other side, there are several studies that show that PKCδ could also act both as a positive regulator of cell growth and as a prosurvival factor in mammary cells. It has been described that PKCδ promotes a mitogenic response throughout the activation of the ERK-MAPK pathway leading to an elevated cyclin D1 expression and an hyperphosphorylated Rb state (*15*). PKCδ also enhances the resistance to apoptotic stimuli, throughout the activation of the Akt pathway and the modulation of NF-κB-dependent gene expression (*14, 15*).

The increase of intracellular Ca^{2+} and DAG production are key steps for the activation of classical PKCs. DAG is a product of the hydrolysis of the phosphatidylinositol-4,5-bisphosphate (PIP2) by the enzyme phospholipase C (PLC) that also produces inositol 1,4,5-triphosphate (IP3) implicated in Ca^{2+} release from the endoplasmic reticulum. When signals are prolonged, DAG production may depend on the activation, by PKC itself, of phospholipase D (PLD), an enzyme that produces phosphatidic acid (PA) from phosphatydilcholine. In turn, PA is converted into DAG by a specific phosphohydrolase. This mechanism is known as biphasic production of DAG, the first phase depending on PLC and the second on PLD (*16*). A pathway involving Src/Ras/RalA is also capable of activating the PLD1 isoform (*17*).

Two of the main PKC downstream events include the activation of MEK/ERK (*18*) and PI3K/Akt pathways (*19*). Atypical PKCs have also been described as activators of MEK/ERK cascade, possibly through a mechanism independent of c-Raf1 that probably involves a direct interaction with MEK (*20*). The PI3K/Akt pathway has been widely implicated in inhibiting apoptotic responses through the phosphorylation of target proteins. Some of these proteins include BAD, inducing the loss of its proapoptotic function, and the activation of NF-κB transcription factor, via the regulation of IκB kinase (IKK), favoring the transcription of prosurvival genes. Thus, the deregulation of PKC expression or activity may lead to enhanced proliferation and/or survival processes that could finally contribute to malignant

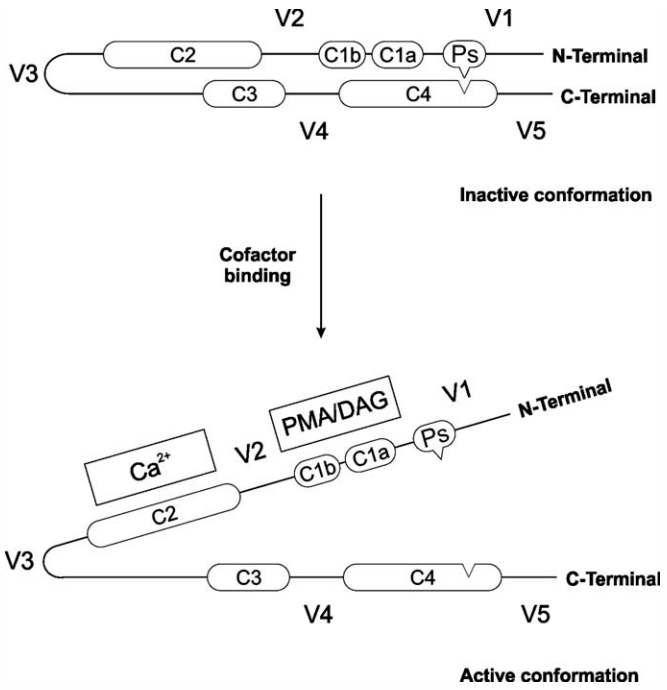

Figure 2. Model of PKC activation by cofactors. In the unstimulated state, PKC adopts a closed spatial conformation where the regulatory domain through the PS sequence interacts with the catalytic domain and prevents the interaction with the substrate. The binding of specific cofactors to the regulatory domain induces conformational changes in the enzyme, exposing the substrate-binding site and allowing activation of the kinase.

transformation-related events. A schematic representation of the activation of classical and novel PKCs and of some of their downstream pathways is illustrated in Fig. 3.

Motility and invasiveness, other key processes involved in cancer progression, are also modulated by PKC expression and/or activation. In this sense, overexpression of PKCα may contribute to increased anchorage-independent growth, tumorigenicity, and metastasis (*10*), whereas overexpression of PKCβII results in increased invasiveness, possibly through increased Ras and MEK activation (*21*). On the other hand, inhibition of PKCε leads to a decrease in motility and invasion as well as to a reduction in tumor growth and metastasis development (*22*). Likewise, PKCε overexpression increases the ability of cells to grow independently of substrate attachment and enhances the incidence and number of lung metastases of breast cancer cells (*23*). PKCδ downregulation has been associated with higher cancer cell invasiveness through the increase in proteases secretion (*24*), and the opposite is observed upon overexpression of this PKC isoform (*25*).

PKC AND BREAST CANCER

Mammary epithelial cells undergo numerous changes during several growth and involution cycles (*26*). These processes include the activation of mitogenic and/or apoptotic signals and it has been described that some PKC isoforms are involved in the control of these pathways. Expression and localization of PKCs are usually modulated during mammary gland differentiation and involution (*27*), and the overexpression of several PKCs has been reported in malignant breast tissue and breast cancer cell lines (*28*). As PKC deregulation is observed in breast cancer (*29*), this kinase family is a promising target for blocking or reverting breast cancer malignancy.

Role of Classical PKC Isoforms in Breast Cancer

PKCα. PKCα has long been recognized to have a role in regulating different aspects of tumor growth and progression (*30*). The role of this isozyme in breast cancer cells is complex, because in some cases, it acts as a tumor promoter, whereas in others, it functions as a tumor suppressor. In this sense, several groups demonstrated that PKCα is overexpressed in human breast cancer cells and in breast tumor samples (*31, 32*), whereas others reported PKCα downregulation (*33*). It is established that increased PKCα expression *in vitro* leads to a more aggressive phenotype (*10*) and it induces tamoxifen (*34*) and multidrug resistance in estrogen receptor (ER)-positive cell lines (*35*). Moreover, the human breast cancer cell line T47-D, which does not express detectable levels of PKCα, has a lower proliferative potential when compared with other PKCα-positive cell lines such as MCF-7, MDA-MB-231, or MDA-MB-468 (*36*). Studies suggest that PKCα activity supports the migratory potential of human breast cancer cells (*16*) arguing for a potential involvement of this PKC isozyme in the modulation of invasion and metastasis. Increasing PKCα levels enhances the migratory potential of MCF-7 (*37*) and MDA-MB-231 (*36*) cells. In addition, PKCα may be also implicated in the induction of epithelial-mesenchymal transition in the highly motile breast cancer cells (*38*).

Using syngeneic models of murine breast cancer, it was demonstrated that a novel and selective PKCα inhibitor aV5-3 almost abrogates metastasis development without affecting the primary tumor growth (*39*). The PKCα antagonistic mechanism of action includes the inhibition of intravasation by reducing matrix metalloproteinase-9 proteolytic activity and also decreasing cell migration. These effects were also accompanied by a reduction in NF-κB activity. Although this drug is in a preclinical phase nowadays, it presents a great potential for the prevention of lung metastasis of patients with breast cancer.

Studies performed using human breast cancer specimens show that PKCα expression associates with several markers of tumor aggressiveness including hormone dependence (*36*). Furthermore, ER-negative human breast cancer cell lines express significantly higher levels of PKCα than ER-positive breast cancer cell lines (*40*). In addition, patients with PKCα-negative tumors have better response to endocrine treatment compared to patients with PKCα-positive tumors (*41*), thus PKCα is a predictive marker of disease outcome under this treatment.

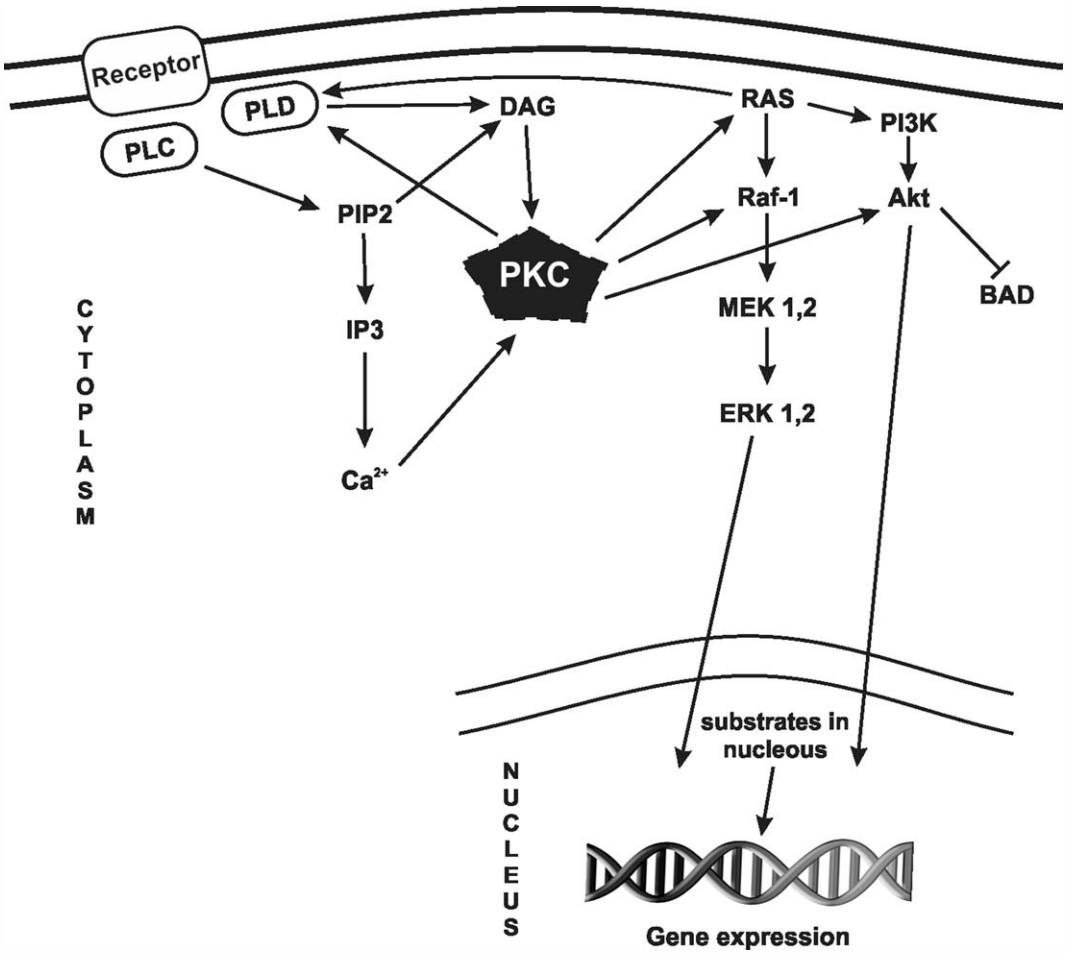

Figure 3. Regulation of classical PKC isoforms. External stimuli activate receptors (tyrosine kinase or G protein-coupled receptors) which are coupled to phospholipase C (PLC) and/or phospholipase D (PLD). PLC cleaves PIP2 into DAG and IP3 whereas PLD indirectly produces DAG. IP3 induces the release of intracellular Ca^{2+}, and both Ca^{2+} and DAG act synergistically in the process of classical PKC activation. PKC is also involved in PLD activation. Some subsequent PKC downstream events include activation of the MAPK and PI3K/Akt pathways.

PKCα has been evaluated as a therapeutic target for breast cancer. This PKC isoform is the target for ISIS 3521 also known as aprinocarsen or LY900003, a 20-mer antisense phosphorothioate oligodeoxynucleotide complementary to the 3′-untranslated region of the mRNA for human PKCα (*42*). ISIS 3521 has been studied as a single agent, as well as in combination with standard chemotherapy, in human patients with different cancers in over 20 trials from phase I to III (*42*). Despite some promising results, no differences from the control group were observed in a small phase II trial in metastatic patients with breast cancer (*43*), suggesting that PKCα may not be an appropriate target for therapy.

PKCβ. In the last years, PKCβ has become an attractive target for breast cancer treatment because this isoform has been described as implicated in mammary tumorigenesis of human and rodent models. PKCβ is generally considered a growth promoter kinase, and the PKCβ-specific inhibitor LY379196 significantly reduces the growth of MCF-7, MDA-MB-231, and BT-474 breast cancer cells (*44*). PKCβ is also a mediator of VEGF-induced endothelial cell proliferation, arguing for a potential involvement of this PKC in angiogenesis.

There is increasing evidence that PKCβ-selective inhibitors are effective in breast cancer both at preclinical and clinical levels (*45*). In this sense, it has been described that the potent PKCβ inhibitor enzastaurin suppresses both tumor growth and tumor-induced angiogenesis in mice bearing breast cancer xenografts (*46*).

Although PKCβ has been mainly described as a growth promoter, overexpression of PKCβ in tumor-derived murine mammary cell lines was shown to cause a significant reduction in tumor growth and metastasis development (*23*). Interestingly, PKCβ overexpression exerts profound inhibitory effects in the production and secretion of proteases involved in invasion and

metastasis as well as it induces the re-expression of fibronectin, a glycoprotein, whose expression was associated with a reduction in the metastatic potential of mammary tumor cells (*47*).

Role of Novel PKC Isoforms in Breast Cancer

PKCδ. The role of PKCδ in breast cancer remains ambiguous, and little information is available regarding expression levels of PKCδ in primary tumors.

Although altered PKCδ expression does not seem to be a prerequisite for breast cancer progression, a few studies including our own have pointed out a protumorigenic role for PKCδ overexpression in murine mammary cells via the induction of survival and anchorage-independent growth (*48*). It has been described that PKCδ can promote proliferation (*15*) and metastasis development (*49*), whereas its depletion is sufficient to drive murine mammary cancer cells into apoptosis (*50*). On the other hand, several studies showed that PKCδ mediates antiproliferative responses. For example, the antimitogenic effect of inositol hexaphosphate in MCF-7 human breast cancer cells, which involves inhibition of ERK and Akt as well as pRb hypophosphorylation, is mediated by PKCδ (*51*). The finding that a small molecule PKCδ inhibitor or a dominant-negative PKCδ mutant impairs phorbol ester-induced arrest in G1 in SKRB-3 breast cancer cells (*52*), further supports this antiproliferative role for PKCδ.

Regarding a potential role for PKCδ as a target for breast cancer therapy, it has been shown that AD198, a novel doxorubicin analog devoid of DNA binding and topoisomerase II inhibitory capacities, induces apoptosis by activating PKCδ (*14*).

Several studies suggest a crosstalk between PKCδ and ER. In fact, ER-positive breast cancer cell lines express considerable amounts of PKCδ and show better endocrine response, whereas ER-negative breast cancer cell lines express low PKCδ levels (*41*). Moreover, PKCδ is likely to play a major role in antiestrogen resistance in breast cancer cells and has been linked with acquired resistance to tamoxifen in patients with breast cancer (*53*).

Interestingly, coexpression of PKCα with PKCδ in cell line models and clinical samples predicts a very short duration of endocrine response and survival. On the other hand, the expression of PKCδ in the absence of PKCα is a predictor of a good endocrine response, while PKCα expression in the absence of PKCδ is associated with ER negativity and endocrine insensitivity (*41*). Assessing the expression levels of these two PKC isoforms may therefore represent a useful predictor marker for patients' responsiveness to endocrine therapy.

PKCε. An oncogenic role has been frequently assigned to PKCε, and it also has been proposed that this PKC is a marker of breast cancer aggressiveness. High PKCε expression levels correlate with tumor grade, HER2 expression, ER negativity, and poor survival in patients with breast cancer (*22*). Moreover, in MDA-MB-231 breast cancer cells, downregulation of PKCε drastically reduces tumor growth and metastasis development [22]. Overexpression of PKCε enhances survival against apoptotic insults and increases the ability of a murine mammary tumor-derived cell line to grow and form colonies in soft agar, suggesting that this novel PKC may have an important role in mammary carcinogenesis and tumor progression. In fact, the same study demonstrated that PKCε expression associates with an increase in both the incidence and the number of spontaneous and experimental lung metastasis on inoculation into syngeneic mice (*23*). Lindemann *et al*. (*54*) have shown that PKCε enhances the proliferative and metastatic capacity of breast cancer cells through the stimulation of parathyroid hormone-related protein expression, which in turn activates the MAPK cascade and the transcription of growth related genes. Overexpression of PKCε was shown to increase total and activated Akt. In addition, an indirect regulation of Akt by PKCε was observed through interactions with integrins (*55*). PKCε also mediates invasion and motility of breast cancer cells. This effect occurs via the activation of Rho GTPases, which contain putative PKC phosphorylation sites (*22, 56*). A speculation is that Rho GTPases could be important PKCε effectors.

Although most studies show that PKCε promotes cell proliferation and survival, a few studies showed that activation of PKCε could contribute to apoptosis. In this regard, it was described that the antiproliferative activity of tamoxifen could be associated with PKCε translocation to membrane (*57*).

PKCη. PKCη is involved in processes associated with mammary gland differentiation. Its expression is upregulated during the transition from a resting to a pregnant state, decreases during lactation, and returns to a level similar to those presented by virgin females during involution (*58*). Estradiol regulates the expression of PKCη in the estrogen responsive cell lines MCF-7 and T47-D. Downregulation of estradiol-induced PKCη expression was observed after treating these cells with progesterone, a hormone involved in the differentiation of the mammary gland (*59*). PKCη has been implicated in the modulation of breast cancer cell proliferation through the modulation of cell-cycle components. In addition, inducible expression of PKCη in MCF-7 cells results in increased cell survival and reduced cleavage of the apoptotic marker PARP-1. Moreover, the activation of caspase-7 and caspase-9 as well as the release of cytochrome *c* are also inhibited by inducible expression of PKCη (*60*).

An increase in the expression levels of PKCη was observed in highly invasive and metastatic human breast tumors, and expression correlates with positive lymph nodes status (*61*). In chemotherapy-treated patients with breast cancer, PKCη expression is reduced in the high-grade tumors. Moreover, PKCη expression correlates with MDR receptors levels (*62*). Thus, PKCη could represent a target for intervention aimed at reducing resistance to anti-cancer treatments.

PKCθ. An important role for PKCθ was found in mammary tumorigenesis, which involves the activation of Akt and derepression of c-Rel transcriptional activity. The activation of c-Rel induces the expression of genes that promote a trans-

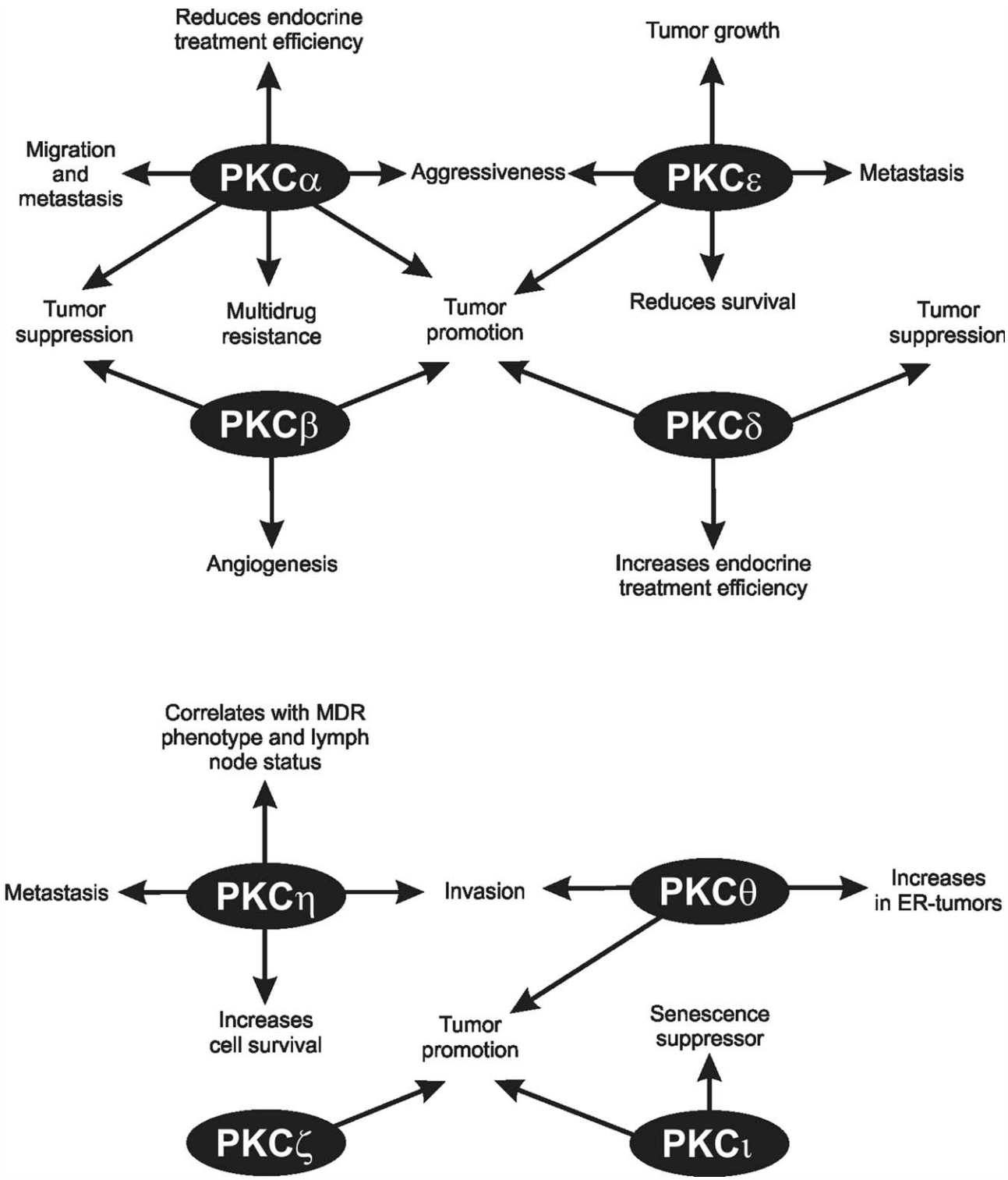

Figure 4. Involvement of PKC isoforms in breast cancer. The figure illustrates specific functions of PKC isozymes in breast cancer cells.

formed phenotype in mammary cells, including cyclin D1, c-Myc, and Bcl-xL, which promote growth and survival, and RelB, which leads to an invasive phenotype (*63*). Normal breast epithelial cells show low PKCθ and Akt1 levels and activities, whereas PKCθ levels are increased particularly in ER-negative human breast cancers (*63*).

Role of Atypical PKC Isoforms in Breast Cancer

The atypical PKC isozymes are structurally and functionally distinct from other PKCs because they lack the calcium-, phospholipid-, and DAG-binding motifs (*12*). It has been described that atypical PKC activity can be regulated by 3-phosphoinositides (*64*) and by phosphoinositide-dependent kinase 1 (PDK1) phosphorylation (*65*). Recent studies have revealed that the N-terminal domain has additional structural motifs involved in the regulation of these PKC isoforms through specific protein–protein interactions, which are important for the subcellular targeting (*66*). In particular, an interaction with PAR-3 and PAR-6 (partitioning defective homolog) proteins, which are involved in the asymmetrical cell division and cell polarization processes as members of a multiprotein complex (*67*), has been described. The main importance of this interaction in cancer progression is that these proteins have a role in the epithelial-to-mesenchymal transition that characterizes the invasive phenotype associated with metastatic carcinomas.

PKCζ. Little information is available regarding PKCζ expression and breast cancer development. Aberrantly expressed PKCζ in mammary cells induces phenotypic alterations associated with malignant transformation and tumor progression. In this regard, we have demonstrated that the stable overexpression of PKCζ in immortalized mammary epithelial cells (NMuMG) activates the mitogenic ERK pathway, leading to profound effects on the ability of NMuMG cells to proliferate, adhere, migrate, and secrete proteases, and that all these effects were dependent on the catalytic activity of the enzyme (*20*). It has also been described that estradiol activates PKCζ in MCF-7 cells, and this PKC isozyme is involved in the cytoplasmic redistribution of p27, which allows G0-arrested cells to re-enter the cell cycle (*68*). It has been postulated that PKCζ is required for human breast cancer cell chemotaxis (*69*), and PKCζ overexpression in MDA-MB-468 stimulates cell motility (*70*). Therefore, these studies link PKCζ to an increase in the proliferative, invasive, and metastatic potential of breast cancer cells.

PKCι. Similarly to PKCε, PKCι was also described as an oncogenic kinase (*66*). PKCι is involved in oncogenic Ras signaling, transformation, and tumorigenicity (*71*). In addition, PKCι is an important downstream mediator in the phosphoinositide pathway (*66*). PKCι expression is upregulated in a subset of breast cancers and breast cancer cell lines. Expression of an oncogenic variant of PI3K (PIK3CA) into breast mammary epithelial cells increases both the expression and activation of PKCι. Furthermore, depletion of PKCι from breast cancer cells increases the number of senescent cells, an effect that is not observed in normal mammary cells. These results suggest that PKCι may function as a suppressor of premature senescence, a mechanism to escape from the first stages of carcinogenesis (*72*). Although PKCι can be used as prognostic marker in non-small cell lung, pancreatic, and ovarian cancer, to our knowledge, there is no information available yet regarding its potential as a marker in breast cancer.

CONCLUDING REMARKS

PKC isozymes are critical players in many signaling pathways involved in the control of cell fate. Thus, alterations in PKC signaling could lead to malignant transformation and tumor progression. Indeed, altered PKC expression and/or activation can be detected in human breast cancer and the expression of some PKC isoforms could be used as prognostic or treatment responsiveness predictive markers. The role of each PKC isoform in the modulation of malignant phenotype in breast cancer is depicted in Fig. 4.

Targeting PKC in the management of breast cancer has become an interesting option. However, a main problem lies in the pleiotropic responses by several PKC isozymes (*e.g.*, PKCδ may be growth stimulatory or growth inhibitory), which is a major limiting factor for the design of isozyme-specific PKC modulators as therapeutic agents. A better understanding of the mechanisms associated with the control of these processes should provide new opportunities for the rational design of PKC modulators as therapeutic agents.

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