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Review

Molecular Mechanisms of Protein Kinase C-induced Apoptosis in Prostate Cancer Cells

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Protein kinase C (PKC) isozymes, a family of serine-threonine kinases, are important regulators of cell proliferation and malignant transformation. Phorbol esters, the prototype PKC activators, cause PKC translocation to the plasma membrane in prostate cancer cells, and trigger an apoptotic response. Studies in recent years have determined that each member of the PKC family exerts different effects on apoptotic or survival pathways. PKCδ, one of the novel PKCs, is a key player of the apoptotic response via the activation of the p38 MAPK pathway. Studies using RNAi revealed that depletion of PKCδ totally abolishes the apoptotic effect of the phorbol ester PMA. Activation of the classical PKCα promotes the dephosphorylation and inactivation of the survival kinase Akt. Studies have assigned a pro-survival role to PKCε, but the function of this PKC isozyme remains controversial. Recently, it has been determined that the PKC apoptotic effect in androgen-dependent prostate cancer cells is mediated by the autocrine secretion of death factors. PKCδ stimulates the release of TNFα from the plasma membrane, and blockade of TNFα secretion or TNFα receptors abrogates the apoptotic response of PMA. Molecular analysis indicates the requirement of the extrinsic apoptotic cascade via the activation of death receptors and caspase-8. Dissecting the pathways downstream of PKC isozymes represents a major challenge to understanding the molecular basis of phorbol ester-induced apoptosis.

Keywords: Apoptosis, Phorbol esters, PKC, Prostate cancer cells, TNFα

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Introduction

Mitogenesis, survival and cell death are mediated by complex signaling events, in which phosphorylation mechanisms play essential roles. Transformation of a normal to a cancerous cell often results from dysregulated signaling pathways leading to hyperactivation of kinases and their effectors, ultimately leading to growth advantage due to uncontrolled proliferation or enhanced survival. One of the important groups of kinases that regulates these processes is protein kinase C (PKC), a family of serine-threonine kinases that have been extensively studied as effectors of tyrosine-kinases and seven transmembrane G-protein-coupled receptors. PKCs comprise at least 10 different isozymes that have been classified into 3 classes based on their structural and biochemical properties: classical PKCs (calcium-dependent cPKCs α, β, and γ), novel PKCs (calcium-independent nPKCs δ, ε, η, and θ), and atypical PKCs (calcium-independent aPKCs ζ and λ/ι,). cPKCs and nPKCs are intracellular targets for diacylglycerol (DAG), a lipid second messenger generated in membranes by phospholipase C isozymes, and for the phorbol esters, agents that activate PKCs via binding of the DAG-binding site (the C1 domain) (Newton, 2003; Yang *et al.*, 2003; Parker *et al.*, 2004).

Cells express multiple PKC isozymes, which could have either overlapping, distinct or even opposite biological functions. As phorbol esters activate cPKCs and nPKCs, the resulting effect will depend on the relative pattern of PKC isozyme expression and the nature of the biological effects mediated by each isozyme. Phorbol ester responses can also be mediated through effectors that do not belong to the PKC family, such as PKDs, RasGRPs, chimaerins, and Munc13s, proteins that have a C1 domain and bind DAG and phorbol esters with high affinity (Kazanietz, 2000; Brose *et al.*, 2002). This represents an additional level of complexity in the phorbol ester responses.

Extensive research in recent decades has implicated PKC

isozymes in tumorigenesis. PKCs are downstream effectors of mitogenic receptors and proto-oncogenes. Phorbol 12-myristate 13-acetate (PMA or TPA) can transform cells that have been "initiated" by overexpression of proto-oncogenes, such as c-Src, suggesting a link between tyrosine kinases and PKC signaling in the development of a transformed phenotype (Lu *et al.*, 1997). Numerous types of cancers show changes in the expression of PKC isozymes, although in most cases a causal relationship between dysregulated PKC expression and malignant transformation has not been demonstrated. For example, prostate cancer tumors show decrease expression of PKCβ and a reciprocal increase in PKCε (Cornford *et al.*, 1999). The limited knowledge on PKC substrates for individual PKCs has represented a major obstacle for understanding the mechanistic basis of PKC-mediated changes in transformation.

PKC isozymes as growth-inhibitory and pro-apoptotic kinases

It has become clear in recent years that PKCs not only promote pro-mitogenic or pro-survival responses, but they can promote cell arrest or lead to cell death through apoptotic mechanisms. Research from several laboratories pointed to distinctive roles for individual PKCs in the control of cell cycle progression. Indeed, PKC isozymes, when activated, can inhibit the cell cycle at multiple levels, both at G1/S and G2/M transitions (Black, 2000; Gavrielides *et al.*, 2004). Hypophosphorylation of retinoblstoma (Rb) and related pocket proteins has been observed in response to PKC activation, which ultimately leads to changes in the expression of E2F-regulated genes (Livneh *et al.*, 1996; Black, 2000). We have recently determined that in lung adenocarcinoma H358 cells PKC activation leads to cell growth arrest. When H358 cells are stimulated with PMA, they do not progress through G1, an effect that involves the up-regulation of the cell cycle inhibitor p21. Analysis of the involvement of PKC isozymes using RNAi revealed that in H358 cells PKCδ mediates p21 up-regulation (both at the mRNA and protein levels), Rb hypophosphorylation and G1 arrest in response to PMA. Overexpression of PKCδ by adenoviral means leads to growth arrest in H358 cells and elevates p21 levels (Nakagawa *et al.*, 2005). In endothelial cells, PKCδ also delays the induction of cyclin D1 following serum stimulation (Ashton *et al.*, 1999). It is also clear that other PKCs can also mediate growth-inhibitory responses, such as PKCα in intestinal cells (Clark *et al.*, 2004), suggesting differential roles depending on the cell context.

In addition to growth arrest, activated PKC isozymes can trigger an apoptotic response in various cellular models. Moreover, PKCs are required for the action of chemotherapeutic drugs, such as etoposide. Early work from Emoto *et al.* (Emoto *et al.*, 1995) revealed that PKCδ was able to cause apoptosis in human U-937 leukemia cells. This unusual response involves caspase-3 cleavage of PKCδ, thus releasing the C-terminal catalytic domain that possesses constitutive kinase activity. This important discovery was subsequently extended to several other cell types, including salivary gland cells, keratinocytes, and neurons (Denning *et al.*, 1998; Reyland *et al.*, 1999; Kaul *et al.*, 2005). Work from the Reyland's laboratory established that PKCδ has a nuclear localization signal and that it translocates to the nucleus. This mechanism may be highly relevant to the apoptotic response, as PKCδ mutants that lack a functional nuclear localization signal have impaired nuclear translocation and cannot induce an apoptotic response (DeVries *et al.*, 2002).

PKC isozymes and apoptosis in prostate cancer cells

Prostate cancer cells have emerged as one of the most studied models for PKC-induced apoptosis. Several laboratories established that PMA treatment triggers an apoptotic response in LNCaP cells (Powell *et al.*, 1996; Zhao *et al.*, 1997; Garzotto *et al.*, 1999; Fujii *et al.*, 2000), a widely used cellular model of androgen-dependent prostate cancer. LNCaP cell death is preceded by p21 up-regulation and Rb dephosphorylation, suggesting that p21 is an important element in this response (Zhao *et al.*, 1997). Interestingly, androgen-independent models do not undergo apoptosis in response to phorbol esters (Powell *et al.*, 1996). Elucidating the molecular basis of phorbol ester-induced apoptosis has become a major area of research due to the therapeutic implications of this response. Deciphering such mechanisms will hopefully underscore factors involved in resistance of androgen-independent cells to apoptosis.

Several important questions have emerged in recent years. One is which PKC isozyme/s mediates the phorbol ester response. Another important question is which signaling pathways mediate the apoptotic effect of phorbol esters. A related issue of great interest is the relative contribution of the extrinsic *vs*. intrinsic apoptotic pathways to the effect and how PKC isozymes modulate these pathways. The intrinsic pathway depends on the depolarization of the mitochondria, and the extrinsic pathway involves the activation of death receptors. The members of the Bcl-2 family are crucial players of the intrinsic cascade through the control of the release of cytochrome c and subsequent activation of caspase-9. The extrinsic pathway is activated by action of death ligands at their plasma membrane receptors, leading to the recruitment of adaptor molecules and caspase-8 and the subsequent activation of downstream caspases. Death receptors can also cause Bid cleavage, which leads to cytochrome c release from mitochondria (Zimmermann *et al.*, 2001; Aggarwal, 2003; Harada *et al.*, 2003). How PKC isozymes modulate such pathways is not fully understood. In androgen-dependent prostate cancer cells it seems that PKCs control the apoptotic cascades at multiple levels in an isozyme-specific manner.

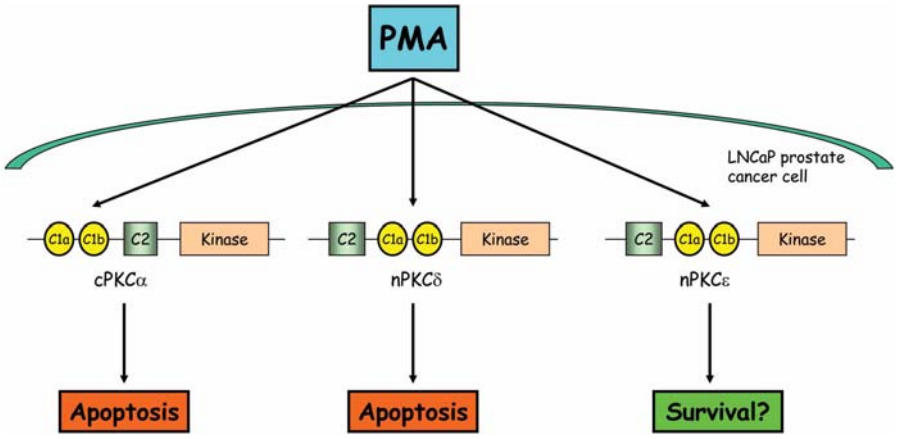

Fig. 1. PKC isozymes and the control of apoptosis and survival in prostate cancer cells. Phorbol esters, such as PMA, bind to the C1 domain in the regulatory region of PKC isozymes to promote their activation. In LNCaP prostate cancer cells, PKCα and PKCδ are pro-apoptotic kinases. The role of PKCε is less clear, but this PKC probably mediates survival signaling.

Role of individual PKC isozymes in phorbol ester-induced apoptosis in prostate cancer cells

Prostate cancer cells express multiple members of the different PKC subgroups. LNCaP cells express the classical PKCα, the novel PKCδ and PKCε, and the atypical PKCζ. In this cell line, different pharmacological and molecular approaches, including isozyme-selective PKC activators and inhibitors, dominant-negative mutants and RNAi, have been used to dissect the role of each PKC in apoptosis and survival (Fig. 1).

PKCα: Early studies conducted in LNCaP cells stably overexpressing PKCα suggested that activation of this isozyme was critical to the PMA apoptotic response (Powell *et al.*, 1996). This work demonstrated a strong correlation between the presence or absence of PKCα in the membrane, and apoptosis induction or resistance, respectively. In androgen-independent PC3 cells, inhibition of PKCα rather appeared to be associated with growth inhibition (Lamm *et al.*, 1997). In the rat Dunning R3327 model, PKCα mediates 12(S)-HETE enhancement of prostate tumor cell invasion (Liu *et al.*, 1994).

Studies from our laboratory in collaboration with the laboratories of Dr. Peter M. Blumberg and Dr. Victor Marquez (NIH) identified a diacylglycerol analogue, HK654, that triggers apoptosis in LNCaP cells by specifically activating PKCα (Garcia-Bermejo *et al.*, 2002). The effect of HK654 could be inhibited by a dominant-negative PKCα mutant. Although this analogue does not discriminate between PKC isozymes *in vitro*, it causes differential intracellular relocalization of PKC isozymes, with PKCα being targeted to the plasma membrane as the main event that correlates with the apoptotic response. Consistently, the overexpression of PKCα using an adenoviral delivery approach potentiates PMA-induced apoptosis. Other PKCs might also mediate PMA-induced apoptosis in LNCaP cells, as the cPKC inhibitor Gö6976 completely blocked the effect of HK654, but only partially blocked the apoptotic effect of PMA (Garcia-Bermejo *et al.*, 2002). This idea is supported by an interesting study in C4-2 prostate cancer cells, an androgen-hypersensitive derivative of the LNCaP cell line (Yin *et al.*, 2005), in which the stable expression of short hairpin RNAs to knock-down specific isozymes suggested that the function of PKCα is redundant and can be replaced with PKCε activity.

PKCδ: Early studies in which this nPKC was delivered into LNCaP cells by adenoviral vectors implicated PKCδ in phorbol ester-induced apoptosis (Fujii *et al.*, 2000). The majority of studies published to date indicate that this nPKC is a major player in apoptosis, not only in prostate cancer cells, but also in other cellular models. We found that overexpression of PKCδ in LNCaP cells causes a reduction in cell number by potentiating the apoptotic effect of PMA. Noteworthy is the fact that unlike other cell types, the induction of LNCaP cell apoptosis by PKCδ does not involve its proteolytic cleavage by caspase-3, suggesting that it depends on allosteric activation of the enzyme upon translocation to the plasma membrane. PMA-induced apoptosis in LNCaP cells can be blocked by a dominant-negative PKCδ or a PKCδ inhibitor (Fujii *et al.*, 2000), as well as by PKCδ RNAi (Gonzalez-Guerrrico *et al.*, 2005). Stable expression of short hairpin RNA against PKCδ inhibited PMA-induced C4-2 cell death (Yin *et al.*, 2005). PKCδ activation in prostate cancer cells plays a key role in the autocrine release of death receptor ligands and the activation of the extrinsic apoptotic cascade (Gonzalez-Guerrrico *et al.*, 2005).

PKCε**:** This nPKC has been shown to promote mitogenicity or survival in many cell types, a fact that is reflected in the correlation between PKCε expression and the progression of many cancers (Perletti *et al.*, 1998; Knauf *et al.*, 1999; Sharif *et al.*, 1999). Studies using prostate cancer cell culture models have been controversial. In DU-145 cells the down-regulation of PKCε apparently prevented apoptosis (Rusnak *et al.*,

1996), although those studies relied on PKC inhibitors which were subsequently shown to target primarily other kinases. Later studies by Terrian and coworkers (Wu *et al.*, 2002a) showed that PKCε overexpression could transform LNCaP cells from an androgen-dependent to an androgen-independent state. Further work established that overexpression of PKCε leads to accelerated proliferation of LNCaP cells due to constitutive activation of the ERK cascade; and in addition, it conferred resistance to phorbol ester-induced apoptosis, the later associated with an inhibition of Bax oligomerization, which is necessary for its mitochondrial integration and cytochrome c release (McJilton *et al.*, 2003). In the CWR22 model of human prostate cancer, PKCε is up-regulated in recurrent tumors and is required to maintain the androgen-independent proliferation of the CWR-R1 cell line (Wu *et al.*, 2002b). Recent studies by Yin *et al*. (Yin *et al.*, 2005) revealed that stable knockdown of PKCε in C4-2 cells did not have any effect on phorbol ester-induced apoptosis, but it had an inhibitory effect when PKCα was simultaneously depleted. Another recent study found that overexpression of PKCε does not alter the sensitivity of LNCaP to either PMA or androgen, but it sensitizes cells to induction of apoptosis by bryostatin 1 (Powell *et al.*, 2005). The apparent contradictory results may be a consequence of the different approaches used.

Signaling pathways involved in PKC-mediated apoptosis

Numerous recent studies have advanced our knowledge on the molecular events that mediate the apoptotic effect of PMA. PKCs are known to regulate multiple pathways, including MAPK cascades (ERK1/2, JNK, p38), the PI3K-Akt pathway, Stats, and NF-kB, although the precise mechanisms involved in this case have not been fully deciphered.

PMA treatment causes a marked increase in phospho-p38 levels in LNCaP cells. p38 inhibitors block the apoptotic effect of PMA, suggesting a role for p38 in PKC-mediated apoptosis (Tanaka *et al.*, 2003). This result has been later extended to C4-2 cells (Yin *et al.*, 2005). Preliminary evidence suggests that both PKCα and PKCδ activate the p38 cascade in LNCaP cells (Fujii *et al.*, 2000). It is not clear whether PKCs phosphorylate and activate kinases upstream from p38.

One rapid event following addition of PMA to LNCaP cells is the phosphorylation of ERK1/2. Inhibition of MEK1, the kinase upstream of ERK1/2, significantly potentiates PMA-induced apoptosis (Tanaka *et al.*, 2003). This is consistent with the notion that the ERK cascade provides survival signals that counterbalance the apoptotic response. Therefore, it is plausible that the balance in the activation of the different MAPK cascades is crucial for determining the cell fate in LNCaP cells.

Phosphorylation of JNK in LNCaP cells was observed shortly after addition of PMA. Initial studies show that the JNK inhibitor SP600125 did not block the apoptotic effect of phorbol esters (Tanaka *et al.*, 2003). Subsequent studies in C4-2 cells showed that inhibition of JNK1 and JNK2 had a small but significant inhibitory effect on PMA-induced cell death (Yin *et al.*, 2005). Ikezoe *et al*. (Ikezoe *et al.*, 2004) showed that SP600125 protects LNCaP cells from growth inhibition mediated by PMA, and that overexpression of JIP-1, a JNK interacting protein and negative regulator of the pathway, attenuates phorbol ester-induced apoptosis (Ikezoe *et al.*, 2004). Recent results from our laboratory revealed that both JNK and p38 activation may be late events due to the autocrine activation of death receptors (see below).

The PI3K-Akt pathway is hyperactivated in LNCaP cells due to loss of PTEN function (Nesterov *et al.*, 2001). PTEN dephosphorylates the lipid products of PI3K, which explains the Akt hyperactivation. Loss of PTEN has been observed in a large percentage of prostate tumor specimens (Steck *et al.*, 1997), suggesting that Akt is a dominant survival pathway in prostate cancer. Remarkably, treatment of LNCaP cells with PMA or the DAG-mimetic HK654 leads to a rapid dephosphorylation of Akt, an effect that correlates with reduced Akt activity. The activity of PI3K and PDK1, upstream regulators of Akt, remains unchanged. Expression of constitutively active Akt (myr-Akt) significantly reduced PMA-induced apoptosis in LNCaP cells, an indication that inactivation of Akt is required for apoptosis. Akt dephosphorylation could be blocked by okadaic acid, suggesting that it may be mediated by a PP2A phosphatase. Studies on isozyme specificity revealed that PKCα is the main player in Akt inactivation (Tanaka *et al.*, 2003).

Phorbol esters induce a rapid increase in ceramide generation, which seem to be required for PMA-induced LNCaP cell death (Garzotto *et al.*, 1999). The effect involves *de novo* ceramide generation through the enzyme ceramide synthase. Acid and neutral sphingomyelinase activities are not enhanced by PMA, but inhibition of ceramide synthase by fumonisin B1 abrogates both ceramide production and PMA-induced apoptosis. A subsequent study revealed that ceramide-induced death is more necrotic than apoptotic (Engedal *et al.*, 2001).

PMA-induced apoptosis of prostate cancer cells requires the activation of NAG-1 (nonsteroidal anti-inflammatory drug activated gene). NAG-1 is a member of the transforming growth factor-beta superfamily that is involved in cellular processes such as inflammation, apoptosis/survival and tumorigenesis. PMA increases NAG-1 mRNA and protein levels in LNCaP cells, an effect that is mediated by binding of NF-kB to the NAG-1 promoter and is potentiated by expression of constitutively active mutants of PKCα or PKCδ. The expression of NAG-1 is independent of p38, JNK, MEK and Akt. Silencing of NAG-1 expression impairs PMA-induced apoptosis in LNCaP cells, suggesting that the induction of NAG-1 negatively affects LNCaP cell survival (Shim *et al.*, 2005). A model illustrating the signaling cascades regulated by PKC in LNCaP is summarized in Fig. 2.

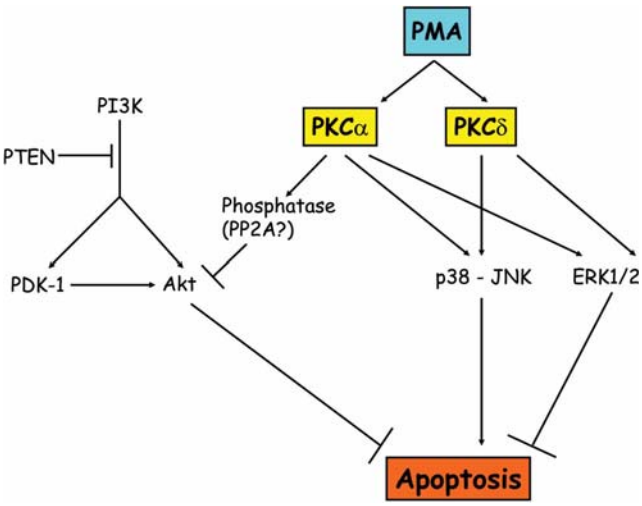

Fig. 2. Signaling pathways involved in PKC-mediated apoptosis. Pro-apoptotic PKCs activate the p38 cascade in LNCaP prostate cancer cells. Inhibition of p38 impairs phorbol ester-induced apoptosis. On the other hand, inhibition of the ERK cascade enhances phorbol ester-induced apoptosis, suggesting opposing roles for these two pathways in prostate cancer cells. Inactivation of the survival kinase Akt occurs when LNCaP cells are treated with phorbol esters. PKCα mediates this effect by activating a phosphatase that dephosphorylates and inactivates Akt.

Identification of a novel PKCδ-mediated pro-apoptotic autocrine loop

PKCs can stimulate the autocrine secretion of mitogenic factors. PKCε overexpression stimulates DNA synthesis in R6 fibroblasts through the secretion of TGFβ (Cacace *et al.*, 1998). Likewise, Caco-2 cell proliferation mediated by PKC involves the stimulation of the IGF-I receptor through an autocrine loop (Cadoret *et al.*, 1998). However, until recently there was no strong evidence that autocrine mechanisms could be involved in PKC-mediated apoptosis. In a recent study we found that conditioned medium collected from LNCaP cells treated with PMA (CM-PMA) induces a strong apoptotic response, similar to that of PMA alone. Apoptosis does not occur when the secreted factors were continuously removed from the medium. The autocrine loop involves at least the secretion of two members of the TNF family, TNFα and TRAIL (Fig. 3), while FasL appears not to be involved. Neutralizing antibodies against TNFα and TRAIL, or against their receptors, as well as depletion of TNFα or TRAIL receptors, block cell death induced by CM-PMA (Gonzalez-Guerrrico *et al.*, 2005).

A combination of TNFα and TRAIL treatment is not sufficient to cause a full apoptotic response in LNCaP cells, suggesting that other factors still unidentified may also be required for the PMA effect. TNFα only causes a partial activation of signaling pathways required for apoptosis (*i.e.*, JNK), in contrast to a sustained activation observed with CM-PMA (Gonzalez-Guerrrico *et al.*, 2005). It has been described that sustained activation of JNK makes TNFα more efficient as a cell killer (Zhang *et al.*, 2004). It might be possible that factors in the CM-PMA sensitize the cells to the apoptotic stimuli, possibly by inhibiting the dominant Akt survival pathway.

PKCδ is a crucial player in the release of autocrine factors. CM-PMA collected from PKCδ-depleted LNCaP cells does not have apoptogenic activity. One possibility is that PKCδ regulates the activity of TACE, the enzyme responsible for TNFα shedding. TACE is indeed required for the apoptotic effect of PMA in LNCaP cells, as either RNAi depletion or pharmacological inhibition of this enzyme with the inhibitor TAPI-2 impairs the apoptotic effect of PMA (Gonzalez-Guerrrico *et al.*, 2005).

Analysis of the signaling events that mediate the effect of

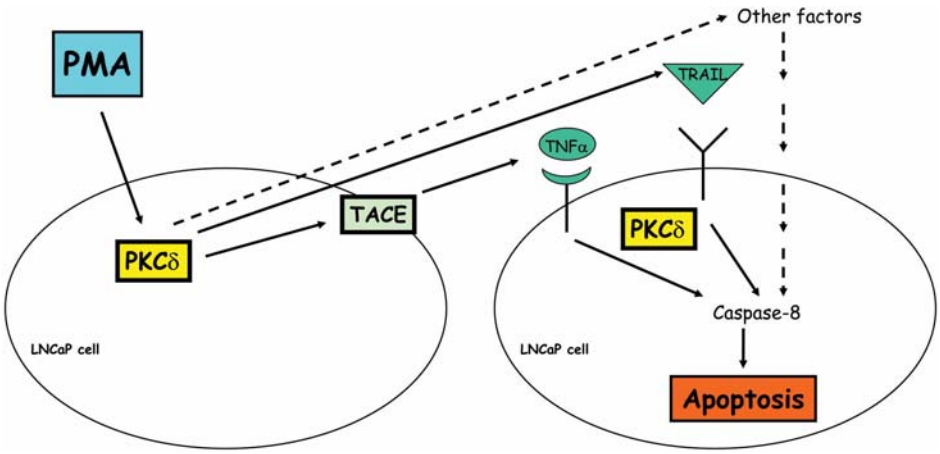

Fig. 3. PKCδ activates the secretion of factors that causes cell death in prostate cancer cells. Factors secreted from PMA-treated prostate cancer cells, which include TNFα and TRAIL, activate death receptors, caspase-8, and the extrinsic apoptotic cascade. Interestingly, PKCδ is also required for the effect of the secreted factors, although it is not clear how PKCδ regulates the extrinsic apoptotic pathway.

CM-PMA revealed that p38, JNK, NF-kB, and caspase-8 become activated by the autocrine secreted factors, suggesting a potential role for the extrinsic apoptotic cascade. RNAi depletion of key mediators of the extrinsic pathway, such as caspase-8 or FADD, resulted in a significant impairment of the apoptotic effect of CM-PMA. It seems that PKCδ also plays a role downstream of the death receptors, since inhibition or RNAi depletion of this nPKC almost completely blocks the apoptotic effect of the CM-PMA. Thus, PKCδ plays a dual role, both in the release of apoptogenic factors as well as an effector of these factors.

Final remarks

Over the past few years our knowledge on the mechanism of how PKC activation controls apoptotic and survival pathways has improved substantially. It is clear that PKCδ is the most relevant apoptotic PKC in prostate cancer cells, as also established in several other cell types. It would be important to establish the relevant substrates of this kinase that mediate the apoptotic response. One attractive target is TACE, the enzyme involved in the release of TNFα. The phorbol ester responses clearly involve the activation of multiple targets and pathways, suggesting a tight control of PKC signaling.

An important finding has been that apoptosis by PMA stimulation occurs through an autocrine loop that involves the activation of death receptors and the extrinsic apoptotic pathway. Most probably the effect is mediated by a number of death factors leading to a combinatorial activation of signaling cascades and therefore a more potent, efficient and controlled apoptotic response. Understanding these mechanisms will have great implications for the identification of therapeutic targets. Ultimately, studies using animal models, such as transgenic or knock-out PKC models, will establish the importance of individual PKC isozymes and their effectors in the control of apoptosis and survival in prostate cancer cells.

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