

# Protein kinase C family: On the crossroads of cell signaling in skin and tumor epithelium

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**Abstract** The protein kinase C (PKC) family represents a large group of phospholipid dependent enzymes catalyzing the covalent transfer of phosphate from ATP to serine and threonine residues of proteins. Phosphorylation of the substrate proteins induces a conformational change resulting in modification of their functional properties. The PKC family consists of at least ten members, divided into three subgroups: classical PKCs ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), and atypical PKCs ( $\zeta$ ,  $\iota/\lambda$ ). The specific cofactor requirements, tissue distribution, and cellular compartmentalization suggest differential functions and fine tuning of specific signaling cascades for each isoform. Thus, specific stimuli can lead to differential responses via isoform specific PKC signaling regulated by their expression, localization, and phosphorylation status in particular biological settings. PKC isoforms are activated by a variety of extracellular signals and, in turn, modify the activities of cellular proteins including receptors, enzymes, cytoskeletal proteins, and transcription factors. Accordingly, the PKC family

plays a central role in cellular signal processing. Accumulating data suggest that various PKC isoforms participate in the regulation of cell proliferation, differentiation, survival and death. These findings have enabled identification of abnormalities in PKC isoform function, as they occur in several cancers. Specifically, the initiation of squamous cell carcinoma formation and progression to the malignant phenotype was found to be associated with distinct changes in PKC expression, activation, distribution, and phosphorylation. These studies were recently further extended to transgenic and knockout animals, which allowed a more direct analysis of individual PKC functions. Accordingly, this review is focused on the involvement of PKC in physiology and pathology of the skin.

## Basic structure and properties of the protein kinase C family

A prototype of the protein kinase C (PKC) family of serine/threonine kinases was first described by Nishizuka and coworkers (Takai et al. 1979), who initially discovered that PKC is activated by diacylglycerol (DAG), a natural degradation product of phosphatidylinositol (Inoue et al. 1977; Kishimoto et al. 1980). Further studies revealed that PKC is the intracellular receptor of tumor promoting phorbol esters (Castagna et al. 1982). So far, ten isoforms were found (Nishizuka 1988) as listed below in Fig. 1. While PKC  $\iota$  and  $\lambda$  represent human/mouse orthologues (Nishizuka 1995), the former 'PKC $\mu$ ' is not included, belonging to a distinct kinase family (PKD; Dekker et al. 1995; Parker and Murray-Rust 2004). PKCs are involved in a large variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation, and cell death (Nishizuka 1989, 1995).

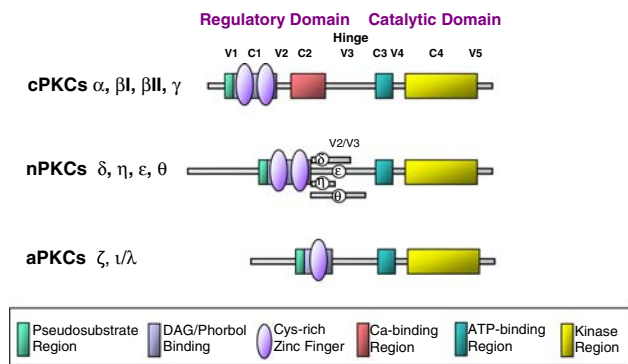
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**Fig. 1** PKC family isoforms

All PKC family members share a structural backbone, mainly consisting of a regulatory domain at the N-terminus and a catalytic domain at the C-terminus. The regions are categorized as conserved regions (C1–C4) and regions that vary between isoforms (V1–V5) (Nishizuka 1988; Kikkawa et al. 1989). Moreover, in common is a pseudosubstrate domain in the regulatory region, closely resembling the substrate recognition motif, which blocks the recognition site and prevents activation (Blumberg 1991; House and Kemp 1987). The PKC isoforms can be divided into three major groups based on their structural characteristics and cofactor requirements (Fig. 1). These include the classical cPKC ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ), novel nPKC ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), and the atypical aPKC ( $\zeta$  and  $\iota/\lambda$ ) isoforms (Azzi et al. 1992; Kikkawa et al. 1989; Svetek et al. 1995). All family members require phosphatidylserine, a component of the phospholipid bilayer, for their activation. Classical cPKCs are calcium ( $\text{Ca}^{2+}$ ) sensitive and need in addition DAG or phorbol esters for activation. The novel nPKCs are  $\text{Ca}^{2+}$  independent but still require DAG or phorbol esters (Kazanietz et al. 1993). For the atypical aPKCs, being also  $\text{Ca}^{2+}$  independent, phosphatidylserine is sufficient for their maximal activity (Chauhan et al. 1990). It should be noted that in nPKCs and aPKCs, the C2 domain of cPKCs ( $\text{Ca}^{2+}$  binding) is replaced by a C2-like domain placed in front of C1 (C1A, C1B; Grner and Kazanietz 2007). The distinct domain structures provide a basis for broad-range but also specific interactions with a large variety of activators and inhibitors, respectively (further reviews: Jaken and Parker 2000; Mellor and Parker 1998; Way et al. 2000).

In this review we will summarize the properties and regulation of the various PKC isoforms and their role in cell signaling. We will then provide a detailed description of the roles played by individual PKC isoforms in skin physiology and cancer pathology. Finally, we present novel genetic approaches used to gain further insights into the contributions of the various PKC isoforms to the development of skin cancer.

## Substrate specificity

One of the major regulatory mechanisms implicated in specific activities of PKC isoforms in cellular signaling is associated with phosphorylation of distinct target substrates. For example, PKC $\alpha$ ,  $\beta$ , and  $\gamma$  are potent kinases for histones, myelin basic protein (MBP), and protamine (Hofmann 1997), whereas PKC $\delta$ ,  $\epsilon$ , and  $\eta$  do not exhibit this activity. Crucial for substrate recognition is the relief of the inhibitory pseudosubstrate region within the regulatory domain. This issue was studied using chimeras as well as by mutational analysis, which confirmed the role of the pseudosubstrate region in the selectivity of PKC $\alpha$ ,  $\epsilon$ , and  $\eta$  (Dekker et al. 1993; Dekker and Parker 1994). Several publications give a detailed overview of the structural aspects and the biochemical properties of PKC isoforms and substrate specificity (Le Good et al. 1998; Newton 1995, 1996; Parekh et al. 2000). Representative examples for typical PKC substrates are STICKs (substrates that interact with C kinase), which are phospholipid-binding proteins. Phosphorylation of STICKs by PKC modifies their activity, reducing their binding to calmodulin and actin (Jaken and Parker 2000). Nevertheless, although various substrates have been linked to distinct PKC isoforms, it became clear that most PKC isoforms phosphorylate similar sequences. As reported in several studies, substrate selectivity is quantitative in nature, where the affinity of each PKC to specific substrates is determined by  $K_m$  values. Thus, other mechanisms must exist to direct these PKC isoforms to distinct signaling pathways. The levels of control include the multiplicity and quantity of PKC isoforms expressed in specific cellular settings, distinct tissue distribution, intracellular compartmentalization mediated by various adapter or scaffolding proteins, and modification of the phosphorylation state of the PKC isoforms and those adapters. Together, these regulatory processes provide the fine-tuning of PKC action in numerous ways, depending on the particular stimuli and the cellular or tissue context, and will be briefly described below.

## Tyrosine phosphorylation

PKC isoforms have a serine/threonine kinase activity, but conversely require serine/threonine phosphorylation for their own activation (reviewed in detail: Le Good and Brindley 2004; Newton 2003; Parekh et al. 2000). However, they are also found to be regulated by tyrosine phosphorylation, which initially has been linked to PKC $\delta$  inhibition (Denning et al. 1993; Denning et al. 1996). Correspondingly, the constitutive phosphorylation of certain tyrosines by src kinases in v- $\text{H}_{\text{a}}$ ras transfected keratinocytes caused permanent inactivation of PKC $\delta$  (Joseloff et al.

2002). But according to the latter and other publications, phosphorylation of tyrosines can also positively regulate the activation of PKC $\delta$  as well as other isoforms, including PKC $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\zeta$ . This has been demonstrated for the response to H<sub>2</sub>O<sub>2</sub> (Konishi et al. 1997), ionizing or UV irradiation (Fukunaga et al. 2001; Uckun et al. 1993: addressing no specific isoform), other inducers of apoptosis (Blass et al. 2002) or certain growth factors (Braiman et al. 1999b; Gschwendt et al. 1994; Li et al. 1994a, b). Thus, the specific effects of tyrosine phosphorylation on the activation of the various PKC isoforms are currently still somewhat controversial. A likely mechanism is that phosphorylation of distinct tyrosine residues induces specific conformational changes depending on the particular PKC isoform (Braiman et al. 2001b; Dutil et al. 1994; Joseloff et al. 2002; Le Good and Brindley 2004; Ohmori et al. 1998). As another explanation, it has been proposed that tyrosine phosphorylation directs PKCs towards specific substrates thereby addressing diverse signaling routes (Gschwendt 1999; Jaken and Parker 2000; Li et al. 1994a; Tapia et al. 2003).

### Intracellular distribution

Another important feature of PKC activation involves the association of PKC isoforms with phospholipids to form stable membrane complexes. This is the basis for the translocation assay as the classical indication for PKC activation. In addition, some PKC isoforms were found to localize to distinct cellular compartments. This includes preferential localization of PKC $\alpha$  at the keratin cytoskeleton, tight junctions, caveolae and desmosome complexes, PKC $\gamma$  within the Golgi apparatus, and of PKC $\eta$  throughout the perinuclear rough endoplasmic reticulum (RER) (Cardell et al. 1998; Jansen et al. 2001a; Lehel et al. 1995). Furthermore, specific translocation of activated PKCs participates in determining of the functional outcome such as induction of transcription. For example, translocation of PKC $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  to mitochondria, the Golgi apparatus, nuclear or perinuclear regions results in regulation of mitosis, apoptosis, and cell survival pathways (Buchner 2000; Denning et al. 2002; DeVries et al. 2002; Li et al. 1999; Lucas and Sanchez-Margalet 1995; Luria et al. 2000; Wang et al. 2004). Moreover, PKC activation in the plasma membrane leads to serine phosphorylation and endocytosis of various transmembrane proteins and receptors. This includes internalization of fibroblast growth factor receptor (FGFR) by PKC $\alpha$  (Asakai et al. 1995) and of insulin receptor by PKC $\delta$  (Braiman et al. 2001b). In addition, PKC $\delta$  phosphorylates insulin receptor substrate-1 (IRS-1) in response to insulin, serving as negative feedback regulation of IR function (Liu et al. 2001), and further the extracellular matrix (ECM) receptor integrin  $\alpha$ 6 $\beta$ 4 (Alt et al. 2004),

thus destabilizing firm adhesion of epidermal basal cells. In the context of cell spreading or migration, interactions of PKC $\epsilon$  were found with  $\beta$ 1-integrins (Berrier et al. 2000), also including PKC- $\beta$ 1-complexes with the cytoskeletal component vimentin (Ivaska et al. 2005). Likewise, PKC $\alpha$  can not only associate with  $\beta$ 1-integrins (Ng et al. 1999; Zhang et al. 2001) and syndecans representing another ECM receptor family (Jaken and Parker 2000; Keum et al. 2004; Yoneda and Couchman 2003), but also with both the Par-3/ASIP (atypical PKC isotype-specific interacting protein) and Par-6 cell polarity proteins, found for PKC $\zeta$  and PKC $\iota/\lambda$  as well (Helfrich et al. 2007; Izumi et al. 1998; Suzuki et al. 2002).

An additional mechanism for specific translocation of PKCs from the cytosol to particulate compartments is associated with receptors for activated/inactive kinases (RACKs/RICKs; Mochly-Rosen and Gordon 1998; Parker and Murray-Rust 2004; Schechtman and Mochly-Rosen 2001). By binding to specific sequences in distinct PKC isoforms, RACKs direct their activation state and define their subcellular distribution. In addition, a variety of other substrates or adapter proteins including PICK1 (protein interacting with C-kinase-1) (Reymond et al. 2005), STICKs (described above), and ZIP (zeta interacting protein; Puls et al. 1997) were found to affect PKC localization in their activated (or resting) state (Jaken and Parker 2000; Mochly-Rosen and Gordon 1998; Moscat and Diaz-Meco 2000; Newton 1996; Schechtman and Mochly-Rosen 2001). Many of these adapter proteins undergo interactions with different proteins or protein complexes such as the PKC interacting, p62 (sequestosome 1; Sanchez et al. 1998) a very versatile protein also involved in NF $\kappa$ B activation (recent reports or reviews on adapter proteins: Masukawa et al. 2006; Moscat et al. 2003,2007; Xu and Xia 2007).

### Tissue distribution

PKC isoforms are ubiquitous, but while some (PKC $\alpha$ ,  $\delta$ , and  $\zeta$ ) are widely expressed in all tissues, other isoforms are expressed in a tissue-specific manner (Mellor and Parker 1998; Nishizuka 1989). PKC $\gamma$  for example is largely confined to brain and neuronal tissue (Cardell et al. 1998; Shimohama et al. 1991) and a shortened transcript of PKC $\zeta$ , PKM $\zeta$  to brain as well (Hernandez et al. 2003). According to earlier reports PKC $\iota$  was restricted to testis and insulin secreting cells (Selbie et al. 1993) and PKC $\theta$  to skeletal muscle and T cells (Berry and Nishizuka 1990; Czerwinski et al. 2005; Osada et al. 1992). However, later on both isoforms were also detected in epidermis; so PKC $\iota$  for example is involved in the formation of tight junction (Helfrich et al. 2007).

The activity of distinct PKC isoforms in various tissues directs their function in a tissue-specific manner. For

instance, PKC $\delta$  was shown to control both proliferation and apoptosis in various cell models (Denning et al. 1998; Lu et al. 1997; Lucas and Sanchez-Margalet 1995; Wertheimer et al. 2001). On the other hand, in the “classical” insulin responsive tissues or cells, muscle, liver, and adipocytes, PKC $\delta$  regulates glucose transport and metabolism (Braiman et al. 1999a, 2001a; Farese et al. 1992). Finally, in C6 glioma cells, PKC $\delta$  is involved in the stimulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger (Chen and Wu 1995).

### PKC signaling in skin epidermis

In skin, continuous renewal of the stratified epidermis is maintained by highly specialized processes leading to the production of the non-viable, cornified squames. This, together with tight junctions in granular layers and lipids derived from secreted lamellar bodies, constitutes the protective water barrier of skin (Helfrich et al. 2007; Langbein et al. 2002; Morita and Miyachi 2003; Suzuki et al. 2002). Proliferating basal cells adhere strongly to the basement membrane forming the dermo-epidermal junction, while cells leaving the basal layer lose their proliferative capability (Alt et al. 2001, 2004; Fuchs and Raghavan 2002). The loss of this cell-extracellular matrix contact is closely associated with the initiation of early spinous differentiation, indicated by keratin 1 (K1) and K10 expression instead of the basal keratins K5 and K14. Further maturation in the upper spinous and granular compartments is characterized by the induction of keratinocyte transglutaminase followed by filaggrin, loricrin, repetin, and other late differentiation markers (Huber et al. 2005). Finally, formation of the rigid cornified envelopes correlates with autolysis of intracellular organelles and programmed cell death, giving rise to the mature squames at the skin surface (Eckert et al. 2005; Fuchs and Raghavan 2002; Koster and Roop 2004; Roop et al. 1987; Watt 1989).

Investigations using murine or human keratinocytes in culture indicated that extracellular Ca<sup>2+</sup> concentration and the degree of cell density are powerful regulators of growth and differentiation in vitro, closely following epidermal maturation patterns in vivo (Breitkreutz et al. 1984, 1993; Denning et al. 1995a; Hennings et al. 1980; Ryle et al. 1989; Yuspa et al. 1989). The early data formed the basis of the initial concept that PKC $\alpha$ , as a Ca<sup>2+</sup> sensitive cPKC, may be a key regulator of the initiation of differentiation, as reviewed recently (Denning 2004). But in follow-up studies, besides low epidermal cPKC $\beta$  levels, additional Ca<sup>2+</sup> independent PKC isoforms were demonstrated to be differentially expressed in the epidermal keratinocytes, namely PKC $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ , and  $\theta$  (Denning et al. 1995a; Dlugosz et al. 1992a; Fisher et al. 1993; Geiges et al. 1995; Papp et al. 2004). This largely corresponded to the patterns in vivo,

being similar in human and mouse epidermis (Parekh et al. 2000; Wang et al. 1993, 1999b). Thus, being abundant in proliferating as well as differentiating keratinocytes in vivo and in vitro, specific roles have been identified for each of these isoforms. Very recently PKC $\iota$  was also detected in mouse epidermis, associated with tight junction complexes (Helfrich et al. 2007). Collectively, manifold PKC associated functions and signaling pathways control normal skin physiology, and consequently aberrations thereof are marking the course of skin tumor formation and progression (Chakravarthy et al. 1995; Denning 2004; Li et al. 2002; Ohba et al. 1998; Shen et al. 2001).

### PKC $\alpha$

PKC $\alpha$ , highly abundant in skin, is the major conventional, Ca<sup>2+</sup> responsive, PKC isoform in epidermis and it was initially the only cPKC detected in the keratinocytes in vitro and in vivo (Dlugosz et al. 1992a; Wang et al. 1993). Thus, the levels of the later found PKC $\beta$  ( $\beta$ I and  $\beta$ II) are much lower in keratinocytes, being mainly confined to the epidermal Langerhans cells (Fisher et al. 1993). Therefore, PKC $\alpha$  had been proposed as a key player in Ca<sup>2+</sup> induced differentiation (Denning et al. 1995a; Dlugosz et al. 1992b). Being in epidermis and mainly restricted to suprabasal layers (Denning et al. 2004), PKC $\alpha$  is involved in cell cycle withdrawal and primarily associated with the keratin cytoskeleton and desmosomal cell-cell junctions (Jansen et al. 2001a; Tibudan et al. 2002). Phosphorylation of keratins, as shown already for the other intermediate filament protein vimentin (Ivaska et al. 2005), may turn out to be an important issue for signaling. Since, upon exposure to the classical PKC activator, TPA (12-O-tetradecanoylphorbol-13-acetate) spinous markers were suppressed, PKC $\alpha$  was thought to be largely responsible for the shift from spinous to granular differentiation as a result of TPA activation (Dlugosz and Yuspa 1993; Lee et al. 1998; Matsui et al. 1992; Punnonen et al. 1993). Indeed, blocking PKC $\alpha$  activity or its synthesis by antisense oligonucleotides abolished granular markers and revived spinous markers like K1 and K10. Likewise, implementation of dominant negative PKC $\alpha$  restored the (late) spinous marker involucrin (Deucher et al. 2002). Accordingly, defective differentiation in skin cancer (Tennenbaum et al. 1993; Tomakidi et al. 2003; also references therein) correlates with elevated PKC $\alpha$  activity, also observed in tumor cells in vitro (Dlugosz et al. 1992a, b; Yang et al. 2003). However, overexpression of PKC $\alpha$  in normal human keratinocytes did not alter their differentiation pattern (Deucher et al. 2002). Furthermore, complexes of PKC $\alpha$ , tetraspanins (TM4SF transmembrane proteins), and  $\beta$ 1-integrins (mainly  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 1) were found after TPA stimulation (Zhang et al. 2001) and it

remains to be determined if these complexes are also present in autochthonous tumors. In this respect, a dramatical expansion of pericellular  $\alpha6\beta4$  (and possibly  $\alpha6\beta1$ ) integrin into suprabasal areas was observed in malignant mouse and human tumor models (Tennenbaum et al. 1993; Tomakidi et al. 1999), which closely correlated to expanded  $\beta1$ -integrin patterns in those tissues (own unpublished data). The influence of PKC $\alpha$  on the cellular traffic and membrane recruitment of  $\beta1$ -integrin during migration (Ng et al. 1999) may well promote both wound reepithelialization and tumor cell invasion. Similarly, several PKCs interact with syndecans (Yoneda and Couchman 2003) and syndecan-4, in particular, binds to the catalytic domain of PKC $\alpha$ , thereby potentiating its kinase activity (Jaken and Parker 2000).

Altogether, PKC $\alpha$  plays a central role in both the induction of granular and suppression of spinous differentiation, as such antagonizing PKC $\delta$ , which gets out of balance in malignancy (Denning 2004; Hornia et al. 1999; Stanwell et al. 1996; Yuspa 1994). Thus, considering its largely suprabasal distribution in normal epidermis, the interactions of PKC $\alpha$  with ECM receptors presumably reflect a response to the impact by wounding or tumorigenesis.

### PKC $\delta$

There is mounting evidence assigning PKC $\delta$  a crucial role in the regulation of cell growth, migration, differentiation, and cell death (Denning et al. 1996; Li et al. 2002; Watanabe et al. 1992; Wertheimer et al. 1998). PKC $\delta$ , an nPKC abundant in a variety of tissues is known to respond to different stimuli by very distinct translocation patterns leading to diverse biological effects. Thus, in human keratinocyte cultures (HaCaT cells) but also in fibroblasts or NIH3T3 cells, contact inhibition of growth was shown to correlate with nuclear translocation of PKC $\delta$ , as seen after short TPA exposure (Dietrich et al. 2001; Heit et al. 2001). In normal epidermis PKC $\delta$  is already acting in basal cells, involved in early onset of differentiation (Denning 2004), and the TPA-induced G1 arrest (after longer exposure) is correlated with PKC $\delta$  translocation to the membrane (Ishino et al. 1998). Apoptosis, induced by higher UV doses for example (Denning et al. 1998), has been associated with generation of the constitutively active catalytic fragment (Denning et al. 2002), collectively addressed as protein kinase M, PKM (Inoue et al. 1977), but also with distinct tyrosine phosphorylation as upon H<sub>2</sub>O<sub>2</sub> treatment (Fukunaga et al. 2001; Konishi et al. 1997). Generally, activation involves redistribution of PKC $\delta$  to various cellular compartments such as mitochondria, the Golgi complex, perinuclear, and nuclear sites (Cross et al. 2000; Dekker and Parker 1994; Denning et al. 2002; Farshori et al. 2003;

Kajimoto et al. 2004; Knutson and Hoenig 1997; Li et al. 1999; Novotny-Diermayr et al. 2002). Especially in epidermal basal cells, upon activation PKC $\delta$  is recruited to the stable matrix adhesion sites (hemidesmosomes facing ECM, i.e. basement membrane), phosphorylating the  $\alpha6$  (Alt et al. 2001; Gimond et al. 1995) or both chains of integrin  $\alpha6\beta4$  (Alt et al. 2004). Getting dissociated,  $\alpha6$  and  $\beta4$  are internalized and deactivated, causing hemidesmosomal disintegration. The concomitant loss of stable adhesion allows the basal cells to migrate along the adjacent ECM (as during epithelial wound repair) or upward into the suprabasal compartment (with onset of cell differentiation).

PKC $\delta$  participates in various cytokine and growth factor associated pathways such as MEK-ERK, raf1, and ras (Farshoni et al. 2003), and interacts with down stream elements linked to cancer progression pathways, including Src, ras, STAT-1 and -3 (Gschwendt et al. 1994; Joseloff et al. 2002; Novotny-Diermayr et al. 2002; Tapia et al. 2003; Uddin et al. 2002). Lastly, at the level of transcriptional control regulation by PKC involves Sp-1 and particularly AP-1 sites (Hashimoto et al. 1990; Rutberg et al. 1996), which represent the response elements for TPA regulated genes (Angel et al. 1987). Thus, PKC $\delta$  increases the levels of c-fos and Jun B, while lowering those of c-jun and fra-1 (Fischer et al. 1993; Rutberg et al. 1996). The heterodimer Fra-2/Jun B has been suggested to participate in silencing of genes not required or being detrimental for cornification (Rutberg et al. 1997).

In keratinocytes, as in some other cell systems, PKC $\delta$  is deactivated by tyrosine phosphorylation following ras transformation and in response to various growth factors such as TGF $\alpha$  and EGF (Denning et al. 1993, 1996, 2000; Joseloff et al. 2002). Conversely, it was demonstrated that tyrosine residues of PKC $\delta$  are dephosphorylated upon insulin induced PKC $\delta$  activation. However, in TPA stimulated murine or UV irradiated transformed human keratinocytes, induction of PKC $\delta$  tyrosine phosphorylation has been also correlated with increased activity (Tapia et al. 2003). Apparently, PKC $\delta$  can be differentially phosphorylated at several distinct tyrosine residues having opposing effects on its activity and the physiological outcome (Fukunaga et al. 2001; Li et al. 1994a; Tapia et al. 2003).

Altogether, these observations suggest that PKC $\delta$  represents one of the key isoforms in signaling pathways of growth factors and cytokines, regulating not only the balance of cell growth and differentiation but also accounting for cell death and tumor suppression.

### PKC $\eta$

PKC $\eta$  was identified initially as a novel PKC isoform predominantly expressed in squamous epithelia. In these

tissues, PKC $\eta$  was restricted to suprabasal layers, suggesting a crucial role in epithelial differentiation (Kuroki et al. 2000; Ohba et al. 1998; Osada et al. 1993). Specifically in epidermis, PKC $\eta$  is primarily distributed to the uppermost granular layer, absent in the spinous layers, and only faintly seen in the inner root sheath of the hair follicle (Koizumi et al. 1993; Osada et al. 1990). Within cells, PKC $\eta$  is uniquely concentrated in the perinuclear region and the rough endoplasmic reticulum, and upon activation becomes associated with the keratin cytoskeleton (Chida et al. 1994). Overexpression of PKC $\eta$  in cultured human keratinocytes induces involucrin and transglutaminase-1, implying a regulatory role in cornified envelope formation (Kashiwagi et al. 2002; Ueda et al. 1996). Furthermore, like PKC $\delta$ , activation of PKC $\eta$  correlates with increased levels of granular differentiation markers (Ohba et al. 1998; Takahashi et al. 1998). Correspondingly, it has been demonstrated that PKC $\eta$  is activated by cholesterol sulfate, which accumulates in upper layers, and that this activation is linked with cell cycle arrest at the G1 phase and expression of granular differentiation markers (Chida et al. 1995; Denning et al. 1995b; Ikuta et al. 1994; Kuroki et al. 2000). Concerning possible mechanisms, several studies suggest that PKC $\eta$  regulates cyclin E levels either by activating its promoter or inhibiting its degradation (Fima et al. 2001; Ishino et al. 1998; Kashiwagi et al. 2000). Alternatively, cell cycle arrest is caused by binding of PKC $\eta$  to the cyclin E/cdk2 complex, affecting its nuclear translocation, or by the p21 phosphorylation by PKC $\eta$ , thus inhibiting cdk2 kinase activity (Kashiwagi et al. 2000; Shtutman et al. 2003). PKC $\eta$  mediated differentiation and growth arrest could be further linked to activation of Fyn, a Src kinase family member, and down modulation of EGFR signaling pathways (Cabodi et al. 2000).

This way, PKC $\eta$  provides an additional level of regulation interacting with cyclin E/Cdk2 or with other control elements to prevent cell cycle progression. Overall, PKC $\eta$  is a major isoform uniquely expressed in squamous epithelia and a key regulator at several steps of the transition from the proliferative to the terminally differentiating state.

### PKC $\epsilon$

PKC $\epsilon$ , also part of the nPKC subfamily, is expressed in basal keratinocytes where increased levels and membrane translocation are linked to proliferation and control of differentiation, though its role in normal skin physiology has not been extensively studied. In skin tumor cell lines as well as in ras transformed keratinocytes, PKC $\epsilon$  transcription remains unchanged (Dlugosz et al. 1992a, b). On the contrary, turning to other cell systems, PKC $\epsilon$  was one of the first isoforms found to participate in the transformation of

fibroblasts, other epithelial, and erythroleukemia cells (Cacace et al. 1994; Mischak et al. 1993). Based on these observations, several pathways critical for cell growth could be linked to PKC $\epsilon$  function. This includes induction of autocrine growth factors, activation of ras associated raf-1 and MAPK signaling (Hamilton et al. 2001), regulation of transcriptional activators such as c-fos and c-jun of the AP-1 family, and of NF $\kappa$ B (Razin et al. 1994). In addition, PKC $\epsilon$  was shown to regulate integrin mediated cell adhesion and spreading (Berrier et al. 2000; Chun et al. 1996) which seems to involve interactions with the intermediate filament cytoskeleton as shown for vimentin (Ivaska et al. 2005). This is further in line with recent data, suggesting that this isoform may be crucial in development of skin cancer (Li et al. 2005). Specifically, overexpression of PKC $\epsilon$  mediates squamous cell carcinoma (SCC) induction elicited either by the two-step DMBA (7,12-dimethylbenz(a)-anthracene)-TPA initiation/promotion protocol (see below “tumorigenesis”) or by repeated UV radiation exposure (Aziz et al. 2007; Verma et al. 2006).

### PKC $\zeta$

The atypical isoform PKC $\zeta$  was shown to exert both growth promoting and inhibitory effects, depending on the cell system. Besides growth regulation, PKC $\zeta$  plays an important role in a variety of other cell functions including migration, matrix assembly, transcriptional activation, glucose uptake and IR-signaling (Bandyopadhyay et al. 1997; Braiman et al. 2001a; Centurione et al. 2003; Fedorov et al. 2002; Le Good et al. 1998; Liu et al. 2001; Martin et al. 2002). No changes in PKC $\zeta$  levels or cellular distribution have been observed during Ca<sup>2+</sup> induced keratinocyte differentiation. However, in cells over-expressing the spinous keratin K10, PKC $\zeta$  becomes activated with acquisition of differentiation and inhibition of cell cycle progression (Nishikawa et al. 1997). The activation of PKC $\zeta$  is associated with reduced expression of cyclin D1, sequestration of Akt/PKB, and impaired PI3 kinase signaling. This pathway was confirmed both in vitro and in transgenic mice overexpressing keratin 10, suggesting a functional interaction between PKC $\zeta$  and keratin cytoskeleton (Paramio et al. 2001). Moreover, benign neoplastic keratinocytes express significantly reduced levels of PKC $\zeta$  transcripts in comparison to normal keratinocytes, though no correlation was found between expression levels and ras activation in the tumorigenic cell lines (Dlugosz et al. 1992a). Nevertheless, down-regulation of PKC $\zeta$  may contribute to skin tumorigenesis by releasing constraints on Akt/PKB activity, proceeding during skin tumor promotion and progression (Segrelles et al. 2006; Wilker et al. 2005). A further significant aspect is the PKC $\zeta$  function in determining of cell polarity (Suzuki et al. 2002),

which is diminished or lost in tumorigenesis. Whereas no direct association of PKC $\zeta$  with tight junction proteins has been found, in contrast to PKC $\iota/\lambda$  (Helfrich et al. 2007), PKC $\zeta$  may contribute to tight junction formation. Accordingly, a shortened form, named PKC $\zeta$ II, representing the regulatory domain and apparently transcribed from a distinct gene, has been found to suppress epithelial polarization (Parkinson et al. 2004). Although missing all the other PKC domains, it has been proposed as a new PKC family member. Under physiological conditions PKC $\zeta$ II presumably facilitates tissue assembly and remodeling, counteracting PKC $\zeta$  by competitive binding to effector targets. This way, it may be a very potent player in epithelial tumor plasticity and invasion as well; consequently PKC $\zeta$ II has been found in several autochthonous tumors (Parkinson et al. 2004).

Still another short form, PKM $\zeta$  consisting of the catalytic, constitutively active domain seems to be exclusively transcribed in brain from an alternative, second promoter located within the gene (Hernandez et al. 2003); its function is apparently devoted to establishing of long term memory.

Overall, PKC $\zeta$  affects several major signaling pathways in the early stages of skin differentiation and is, together with PKC  $\iota/\lambda$ , certainly indispensable for architecture and barrier function of squamous epithelia.

### Role of specific PKC isoforms in skin tumorigenesis

The contribution of PKC signaling in the etiology of skin cancer was initially discovered in the classical two-stage chemical carcinogenesis model in mice. The common two-step protocol in chemical carcinogenesis is based on an initiation step, using a low dose of the carcinogen DMBA, and a subsequent promotion phase with repeated applications of a tumor promoter, usually the phorbol ester TPA (Hecker 1985; Hii et al. 1990; Mills and Smart 1989; for a recent comprehensive review: Griner and Kazanietz 2007). Initially, this leads to the formation of benign skin tumors (papillomas) followed by progression to malignant SCCs. Early studies searching for the molecular targets of chemical carcinogenesis revealed that phorbol esters are in fact broad-spectrum activators of the classical and novel PKC isoforms (Hansen et al. 1990; Hecker 1985; Hii et al. 1990; Imamoto et al. 1993; Krauter et al. 1996; Mills et al. 1993). Short TPA exposure induces PKC activation accompanied by translocation of PKC isoforms from the cytosol to membrane or cytoskeletal compartments. Further exposure to TPA, but also other impacts like ionizing or UV radiation, can induce the release of the catalytic PKC domain (PKM) from the intramolecular inhibitory pseudosubstrate domain, resulting in constitutive activation (Denning et al. 1998;

Emoto et al. 1995; Inoue et al. 1977). The proteolytic processing, converting PKCs to PKMs is frequently mediated by calpains, calcium activated proteases (Al and Cohen 1993; Cressman et al. 1995; Kishimoto et al. 1989), but also caspases (Akkaraju and Basu 2000; Datta et al. 1997; Denning et al. 2002) or the interleukin-1 $\beta$  converting enzyme ICE (Emoto et al. 1995).

The positive correlation between the tumor-promoting effects of various phorbol esters with their ability to activate certain PKC isoforms further indicated that PKC activation is a critical step in skin tumor promotion. In concordance with these results, diacylglycerol (DAG), the endogenous activator of PKC and competing with TPA binding (Sharkey et al. 1984), was found to promote tumor formation in mouse skin (Mills and Smart 1989; Mills et al. 1993; Verma 1988). Compared to the stable TPA, however, DAG is turned over very rapidly and physiological concentrations seem to be extremely low.

One hallmark of early events in tumor promotion is the induction of ornithin decarboxylase (ODC), representing the key enzyme in the synthesis of polyamines, which in turn may enhance specific PKC effects, for example, on growth or apoptosis. The TPA triggered increase of ODC is apparently mediated by various PKCs (Jansen et al. 2001a) and has been a consistent finding for murine keratinocytes *in vivo* and *in vitro*, being also observed in organ culture of human skin (Fischer et al. 1993; Hashimoto et al. 1990; Verma et al. 1985, 1986). However, by a direct comparison under serum-free conditions, ODC was raised by TPA, together with c-jun and c-fos, members of the AP-1 transcription factor family (Angel et al. 1987) only in murine keratinocyte cultures. Contrastingly, the human cells required for a corresponding response various growth factors instead (Fischer et al. 1993). These discrepancies may reflect the profound species differences in skin physiology, paralleled by the strikingly distinct morphology. The differences *in vivo* are paralleled *in vitro* by various additional criteria, mostly those related to keratinocyte differentiation (compare Ryle et al. 1989; Watt 1989; Yuspa et al. 1989). Nevertheless, mouse strain specific differences in TPA promotion experiments have also been found, being not always consistent with ODC induction either (Coghlan et al. 2000; Imamoto et al. 1993; Mills and Smart 1989). In several studies ODC levels did neither correspond to tumor yield nor overall PKC activity (Imamoto et al. 1993; Jansen et al. 2001a; Wheeler et al. 2002). One such case is the lacking correlation of ODC induction by PKC $\delta$  and the tumor suppressive function of this isoform (Wheeler et al. 2002).

Conversely, it has been reported that prolonged exposure of keratinocytes to active phorbol esters *in vitro* and *in vivo*, leads to downregulation of PKC activity (Hansen et al. 1990), for example, via proceeding degradation of

PKM (catalytic subunit) by calpains (Al and Cohen 1993; Kishimoto et al. 1989). Like for activation this may involve other internal proteases like caspases (Akkaraju and Basu 2000; Datta et al. 1997; Denning et al. 1998) or turnover via the ubiquitinylation/proteasome pathway (Griner and Kazanietz 2007). Proteolysis by calpains can also release regulatory domains, which may cause PKC inhibition by competitive binding of substrates (Parissenti et al. 1998). Therefore, both activation as well as downregulation of specific PKC isoforms, such as PKC $\delta$  (mediating preventing cell cycle withdrawal) was linked to tumor promotion and progression.

Acute TPA exposure increases particulate PKC $\alpha$  activity in epidermis, while constitutive PKC $\alpha$  activation is observed in skin carcinomas. This is linked to delayed epidermal differentiation, particularly inhibition of spinous markers seen in other SCCs as well (Tennenbaum et al. 1993; Tomakidi et al. 2003). Conversely, both novel and atypical PKC isoforms are reduced in certain stages of skin tumor progression. Reduced levels of PKC $\delta$  activity are seen in benign papillomas *in vivo* and in human SCCs (D'Costa et al. 2006). Moreover, reduction in PKC $\delta$  expression and activity has been described in other epithelial tumors such as bladder and lung carcinomas correlating with a progressive, metastatic tumor phenotype (Leibersperger et al. 1991; Mills and Smart 1989; Wang et al. 1993), though an opposing effect has been reported for metastasizing mammary carcinoma cells (Kiley et al. 1999). Likewise a decrease of PKC $\eta$  enhances tumorigenesis (Chida et al. 1995), lower levels being also characteristic of squamous tumors originating from skin, bladder, and breast with increased malignant potential. Nevertheless, similarly to normal epidermis, skin papillomas and carcinomas revealed residual expression of PKC $\eta$  in differentiating layers (Koizumi et al. 1993). In concordance with these observations, PKC $\eta$  activation appears to counteract skin tumor promotion (Greco et al. 2003; Masso-Welch et al. 2001). But it is important to note that overexpression of PKC $\eta$  in other epidermal resident cells, the melanocytes, was associated with increased proliferation and malignant potential (Akkaraju and Basu 2000).

While expression and activation patterns of PKC $\epsilon$  and PKC $\zeta$  in tumorigenesis *in vivo* have not been studied extensively, both these isoforms were shown to be drastically reduced in some squamous tumors (Verma et al. 2006) or antagonized by increased PKC $\zeta$ II levels (Parkinson et al. 2004). Nonetheless, growth stimulation by PKC $\zeta$  via MAP kinase has also been documented for head and neck SCCs (Cohen et al. 2006).

Typical PKCs seem to be downregulated in basal cell carcinomas (BCC) such as PKC $\alpha$  and PKC $\delta$ . In these tumors, mostly derived from outer root sheath cells (hair follicle "stem cell niche"), the sonic Hedgehog pathway is

aberrantly activated involving the transcription factors Gli-1, -2, and -3. So far it could be demonstrated experimentally that PKC $\alpha$  is suppressing Gli1 activity and thus blocking proliferation of BCC cells (Neill et al. 2003).

Taken together, accumulating data provide strong evidence that the activation of some TPA dependent PKCs, especially of PKC $\alpha$  and PKC $\epsilon$ , and the deactivation of others, like PKC $\delta$  and PKC $\eta$ , is crucial for the induction of tumorigenesis and for tumor progression (Efimova et al. 2002; Fagerstrom et al. 1996; Shimao et al. 1999; Wheeler et al. 2005). In addition concerning tumor therapy, PKC $\alpha$  for example could also mediate multidrug resistance as recently demonstrated in human leukemia cells and in some carcinoma cell lines (Gariboldi et al. 2001, 2003).

### Knockout and transgenic mouse models

The contribution of specific PKC isoforms to physiologic and pathologic processes in skin, including tumor formation and malignant progression, was recently studied by their overexpression or suppression in knockout and transgenic mice (Jansen et al. 2001a; Leitges et al. 2001; Parker and Muray-Rust 2004; Reddig et al. 1999). Skin tumors were induced in these mice utilizing the two-stage carcinogenesis model described above (Griner and Kazanietz 2007). While TPA induced skin hyperplasia in mice overexpressing any of the PKC isoforms, each PKC isoform had specific consequences for the outcome of the tumor phenotype as follows (briefly summarized in Table 1).

#### PKC $\alpha$

Overexpression of PKC $\alpha$  in transgenic mice did not directly affect the epidermis but induced primarily a striking inflammatory response, increased epidermal thickening and edema correlated to neutrophil infiltration, multiple micro-abscesses, and a marked increase of inflammatory cytokines and chemokines, such as COX-2 or macrophage inflammatory protein (MIP) (Wang and Smart 1999). Treatment with TPA caused epidermal hyperplasia and intra-epidermal inflammation, furthermore massive apoptosis (Cataisson et al. 2003; Jansen et al. 2001a). But surprisingly, though PKC $\alpha$  is activated in transformed keratinocytes and in a variety of tumors, PKC $\alpha$  overexpression did not have a significant effect on tumor promotion or progression towards malignancy (Wang and Smart 1999). Nevertheless, persisting hyperplasia and infiltration of inflammatory cells is certainly a risk factor in human skin carcinogenesis over a wider time frame, representing just one of the inherent limitations of mouse tumor models.

**Table 1** Role of PKC isoforms in epidermis, skin pathology and cancer

PKC isoform	Regulatory roles in skin physiology	Activation state in transformed keratinocytes	Skin pathology and cancer in transgenic and <i>knockout</i> -mice
PKC $\alpha$	Induction of granular, inhibition of spinous differentiation	Elevated expression and activity	Increased inflammatory response in epidermis ( <i>Tumor promotion</i> <sup>ab</sup> )
PKC $\delta$	Migration, proliferation, apoptosis	Reduced expression and activity.	Tumor suppression ( <i>Tumor promotion and inhibited apoptosis</i> <sup>a</sup> )
PKC $\eta$	Cornified envelopes, growth arrest and cell cycle regulation	Reduced expression	N.D. ( <i>Tumor promotion</i> <sup>a</sup> )
PKC $\epsilon$	Cell adhesion and spreading	Elevated expression	Reduced papilloma and increased SCC formation, malignant progression
PKC $\zeta$	Early stage of skin differentiation	Reduced expression	N.D.

Effects in transformed cells, transgenic, and knockout<sup>a</sup> mice (<sup>a</sup> in paranthesis) (<sup>b</sup> Hara et al. 2005)

### PKC $\delta$

In contrast, PKC $\delta$  overexpressing mice are extremely resistant to chemically induced tumorigenesis in skin, supporting the role of PKC $\delta$  in cancer suppression. Thus, the incidence of benign papillomas is reduced and progression towards malignancy slowed down dramatically (Reddig et al. 1999), though this may refer merely to chemically and not UV radiation-induced carcinogenesis (Aziz et al. 2006). On the contrary, in mice depleted of PKC $\delta$  apoptosis was suppressed which may enhance tumorigenesis (Humphries et al. 2006). In fact, this confirmed the anti-promoting function of PKC $\delta$  shown in a cell model previously (Lu et al. 1997). Finally, the role of PKC $\delta$  in establishing immune tolerance, which was demonstrated in transgenic mice (Mecklenbrauker et al. 2002), may imply that this isoform could be critical for cell-mediated immunity including tumor cell surveillance.

### PKC $\eta$

While overexpression of PKC $\eta$  may be difficult to accomplish in vivo (no published data available), deficient mice show prolonged epidermal hyperplasia upon TPA treatment and an increased susceptibility to tumor formation. Thus, PKC $\eta$  is supposed to strengthen resistance against tumors (Chida et al. 2003) as previously demonstrated for PKC $\delta$ .

### PKC $\epsilon$

One of the most intriguing models with respect to cancer progression is associated with overexpression of PKC $\epsilon$ . The skin phenotype of PKC $\epsilon$  overexpressing mice is characterized by epidermal hyperproliferation and skin ulceration, beginning at four months of age. However, when tumor formation was induced using a two-stage skin carcinogenesis protocol, papilloma formation was lowered, while progression to SCCs was increased (Jansen et al. 2001b; Reddig

et al. 2000). Furthermore, resulting SCCs appeared highly metastatic as compared to those seen in control littermates (Jansen et al. 2001b). Carcinomas could also arise independent of papillomas (Li et al. 2005) and further the skin of PKC $\epsilon$  overexpressing mice was sensitized to UV radiation-induced SCC formation (Aziz et al. 2007). These results clearly illustrate the complexity and importance of the role of PKC $\epsilon$  in normal skin development as well as in tumor formation and malignant progression.

### Summary and future prospects

Taken together, the accumulating data on distinct PKC functions suggest considerable isoform specificity in PKC signaling which largely affects skin physiology and the development of cancer. Enhancing the complexity, the combined effects of the various PKC isoforms can result in diverse, conflicting consequences. The final outcome depends on tissue specificity, the levels of PKC expression, and the relative contribution of the different PKC isoforms in a given cellular context. Regarding the influence of the microenvironment on the epithelial phenotype, tumor-stroma interactions are still poorly understood. For this purpose the combination of genetically altered epithelial and stromal cells, for example in three-dimensional skin-organotypic coculture (Maas-Szabowski et al. 2001; Nischt et al. 2007), may allow to analyze PKC signaling under more closely defined conditions, mimicking activation during tumor growth and invasion. Finally, the recent approach utilizing transgenic mice with conditional overexpression or suppression of distinct PKC isoforms (i.e. in a tissue specific manner) shall further clarify the contribution of each PKC family member for the dynamics of skin cancer development. In the near future experimental conditions may be generated, which more closely resemble the situation in patients. Such an alternative animal model may provide a SCID (severe combined immune deficiency) mouse, “humanized” by installing a

partial or complete human immune system. By challenging with human tumor cells, this should give rise to a more specific inflammatory cell infiltrate as part of the tumor stroma reaction (Mueller 2006).

The synopsis of all these approaches should eventually disclose the dynamics of PKC-signaling and their relationship to other regulatory networks. Of particular interest in this context will be the crosstalk with further DAG mediated pathways (Griner and Kazanietz 2007) as well as interactions with the large variety of specific substrate and adapter proteins (Moscat et al. 2007). At this point it has to be noted that some effects, formerly assigned to PKCs, are actually mediated by those other DAG receptors, all sharing with PKCs the C1 domain responsible for DAG binding. Such DAG effector molecules include PKDs (protein kinase D1, 2, 3), DGKs (DAG kinases  $\beta$  and  $\gamma$ ), and chimaerins ( $\alpha 1$ , 2 and  $\beta 1$ , 2) (Griner and Kazanietz 2007; Wang et al. 2003, 2006). Nonetheless, this does not exclude the additional modification of some involved adapter proteins by PKCs, thus bridging the individual pathways in the entire frame of signaling networks.

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