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Protein kinase C isozymes and the regulation of diverse cell responses

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Dempsey, Edward C., Alexandra C. Newton, Daria Mochly-Rosen, Alan P. Fields, Mary E. Reyland, Paul A. Insel, and Robert O. Messing. Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol Lung Cell Mol Physiol* 279: L429–L438, 2000.—Individual protein kinase C (PKC) isozymes have been implicated in many cellular responses important in lung health and disease, including permeability, contraction, migration, hypertrophy, proliferation, apoptosis, and secretion. New ideas on mechanisms that regulate PKC activity, including the identification of a novel PKC kinase, 3-phosphoinositide-dependent kinase-1 (PDK-1), that regulates phosphorylation of PKC, have been advanced. The importance of targeted translocation of PKC and isozyme-specific binding proteins (like receptors for activated C-kinase and caveolins) is well established. Phosphorylation state and localization are now thought to be key determinants of isozyme activity and specificity. New concepts on the role of individual PKC isozymes in proliferation and apoptosis are emerging. Opposing roles for selected isozymes in the same cell system have been defined. Coupling to the Wnt signaling pathway has been described. Phenotypes for PKC knockout mice have recently been reported. More specific approaches for studying PKC isozymes and their role in cell responses have been developed. Strengths and weaknesses of different experimental strategies are reviewed. Future directions for investigation are identified.

pulmonary disease; proliferation; apoptosis; 3-phosphoinositide-dependent kinase-1; receptor for activated C-kinase; transgenic mice

HOW INDIVIDUAL ISOZYMES of an enzyme family contribute to the regulation of diverse cell responses is an important area of signal transduction research. One of the most complex and important of these enzyme families is protein kinase C (PKC).

IMPORTANCE OF PKC ISOZYMES IN LUNG DISEASE[1]

Remarkable heterogeneity exists within the PKC signal transduction pathway (Fig. 1). Twelve different isozymes have now been described (29). Individual isozymes have been implicated in many cellular responses important in both normal lung function and the pathogenesis of pulmonary disease. These responses include permeability, contraction, migration,

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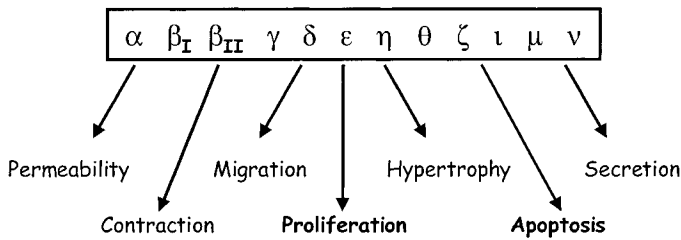
[1] Presented by E. C. Dempsey.

Fig. 1. Heterogeneity within the protein kinase (PK) C signal transduction pathway. The isozymes are indicated in the box. They can be divided into subgroups based on structure and cofactor requirements: conventional (α, β_I, β_{II}, γ), novel (δ, ϵ, η, θ), atypical (ζ, ι), and recently described (μ, ν).

hypertrophy, proliferation, apoptosis, and secretion (1, 4, 12–15, 27, 38–40, 53, 55, 65, 71). These studies strongly suggest that PKC isozymes are important in clinical disorders like pulmonary edema, adult respiratory distress syndrome, interstitial lung disease, asthma, and pulmonary hypertension. In this symposium, the focus is mainly on two cell responses, proliferation and apoptosis (18, 23, 25, 41, 46, 48, 49, 68), that play a major role in the development and eventual regression of the abnormal structural changes observed in these clinical settings.

Why is this area of investigation important? If we can better understand how individual isozymes contribute to the pathogenesis of lung diseases, then we are more likely to find settings where these isozymes will emerge as viable therapeutic targets (18, 24, 44). In this symposium, we also examine the role of selected PKC isozymes in an array of pathological settings outside the lung, including cardiac ischemia (25), carcinogenesis (47), drug-induced cell injury (56), and behavior abnormalities (31, 33). The integrated approaches used and novel findings shown should provide additional insights into how PKC isozymes may contribute to normal lung biology and the pathogenesis of several important pulmonary disorders.

CURRENT APPROACHES FOR INVESTIGATION OF PKC ISOZYMES[2]

Many complementary approaches for investigating the biology of PKC are now available. The first is to test how diverse stimuli activate individual PKC isozymes (34). In the lung, these stimuli include inhaled irritants; mechanical forces; hypoxia; mitogens, vasoconstrictors, or vasodilators; inflammatory mediators; and matrix proteins (12, 15, 16, 38, 39, 53, 54). The phosphorylation state of isozymes is a critical determinant of activity (6, 11, 19, 20, 36). Expression level and activity may or may not correlate. Isozymes can be present in an inactive form. Traditionally, isozyme activation is detected by measuring intracellular translocation to membrane or cytoskeleton. Translocation to the membrane can be detected by Western blotting and measurement of catalytic activity after subcellular fractionation or immunostaining of intact

cells. Sensitivity of the assay is dependent on the affinity of the antibody; thus a negative result does not necessarily mean an isozyme is not present. Specificity of antibody preparations is variable; therefore, appropriate controls (blocking peptides, purified standards) are needed. Increasingly, immunoprecipitation-based kinase assays are being used to evaluate the activity of individual isozymes (56). Antibodies that detect the activated (i.e., phosphorylated) form of a few PKC isozymes have recently been described (51) but are not yet commercially available.

The second approach is to relate the expression pattern of PKC to cell phenotype. The level of any one isozyme in the cell represents a balance between expression and degradation. This balance may be altered in settings of cellular stress or injury (39, 47, 71). Techniques available include Northern and Western blotting with isozyme-specific probes to measure levels, immunostaining with confocal imaging to localize, and comparative and overexpression studies to explore potential roles in cell function. A major concept emerging here is the importance of localization as a determinant of isozyme specificity (3, 17, 45, 52, 58). Receptors for activated C-kinase (RACKs) and caveolins contribute to an elaborate level of intracellular organization and compartmentalization of signaling molecules like PKC. The localization facilitates cross talk between different signaling intermediates, targeted substrate phosphorylation, and regulation of catalytic activity. Analysis of comparative studies can be complicated when the cells of interest grow at different rates, as is often the case. Isozyme expression can change as a function of cell cycle and density; these variables need to be factored into the experimental design and interpretation. Overexpression of one isozyme can alter the levels of others (69). Which isoform is responsible for the change in phenotype may be difficult to discern without more experiments (32). These findings suggest that interdependence between isozymes exists and may be important (57). This concept is not yet well appreciated.

To further implicate individual isozymes in specific cell responses, an array of agonist and antagonist strategies with varying degrees of specificity have been developed. These techniques include pretreatment for 4–24 h with a high concentration of phorbol ester (13, 71), application of inhibitors targeting either the catalytic or regulatory domain (26), or translocation itself (25, 61) and the introduction of antisense RNA or dominant negative proteins via transfection or adenoviral infection (8, 59, 70). Because all of these strategies have limitations, it is usually best to use complementary approaches. An approach needs to be validated when there are questions of inhibitor specificity and when cell type specific effects have been observed, as is the case with phorbol ester-induced downregulation. The downregulatory effects of pretreatment with phorbol ester vary depending on isozyme and cell type (13, 71). A new family of phorbol ester binding proteins (β2-chimaerin) also needs to be considered when interpreting the data. Inhibitors like Go-6976 that target the catalytic domain are competitive with ATP. With this compound and similar derivatives, the IC_{50} for purified
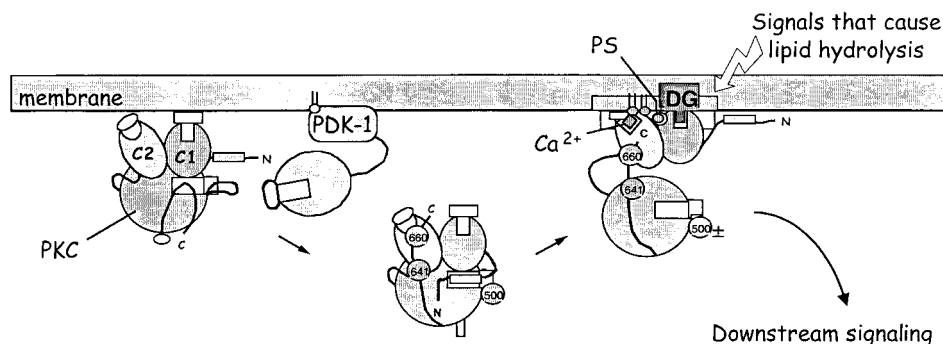
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[2] Presented by E. C. Dempsey.

Fig. 2. Schematic representation of the 2 modes of regulation of PKC: *1*) phosphorylation triggered by 3-phospho-inositide-dependent protein kinase-1 (PDK-1) and *2*) allosteric control mediated by the lipid second messenger diacylglycerol (DG). Unphosphorylated PKC is associated with the membrane where it is phosphorylated by PDK-1 on its activation loop. The immediate consequence of this phosphorylation is the autophosphorylation at two key positions in the carboxy terminus (C1 and C2). The phosphorylated enzyme is then released into the cytosol where it is maintained in an inactive conformation by binding an autoinhibitory sequence, the pseudosubstrate. Membrane recruitment in response to diacylglycerol and phosphatidylserine (PS) provides the energy to expel the pseudosubstrate sequence from the substrate-binding cavity, thus activating PKC for downstream signaling. In addition to the mechanisms discussed here, localization by protein-protein interactions plays a key role in presenting PKC to its upstream and downstream regulators. C, carboxy terminus; N, amino terminus; nos. in circles, phosphorylation motifs.

PKC may be lower than for PKC in intact cells because intracellular ATP concentrations may be higher than those present in the original in vitro assays (13, 26). Myristolation allows introduction of pseudosubstrate peptides into viable cells (21). New strategies have recently been described to facilitate permeabilization of peptide translocation inhibitors (18, 61). These isozyme-specific antagonist strategies complement other pharmacological approaches.

The final, more integrated approach is the use of isolated organ preparations and whole animal models. Eventually, one would like to use both pharmacological strategies and emerging transgenic models (18, 37, 42, 62, 64). Drug specificity and dosing are key issues for the pharmacological studies. Inhibitors can help prove that the phenotype observed with a null transgenic mouse is due to the lack of the gene product and not the consequence of a developmental change. The availability of conditional knockout models can also help address this issue. The use of transgenic models to study intracellular kinases in the lung has been slowed by the limited options currently available for organ-specific targeting of gene manipulations (coupled to the surfactant protein C promoter; specific for only one lung cell type, the type 2 cell). In summary, isolated organ preparations and whole animal studies are powerful approaches but require careful interpretation because the drugs being used probably have more effects than we think and manipulation of PKC genes can potentially alter levels of other key cell intermediates.

NEW IDEAS ON MECHANISMS THAT REGULATE PKC ACTIVITY[3]

PKC is regulated by two sequential, and equally critical, mechanisms: *1*) phosphorylation triggered by the recently discovered 3-phosphoinositide-dependent kinase (PDK)-1 and *2*) binding to the lipid second messenger diacylglycerol (Fig. 2). Each mechanism regulates the structure, subcellular localization, and function of PKC. This contribution focuses on recent advances in understanding the regulation of PKC by its upstream kinase.

PDK-1 is the upstream kinase for the activation loop of PKCs. The first event in the regulation of PKC is phosphorylation of newly synthesized protein at three conserved positions within the catalytic domain. The first rate-limiting phosphorylation occurs on a segment at the entrance to the active site referred to as the activation loop. Negative charge at this phosphorylation site regulates the function of diverse members of the kinase superfamily, including both tyrosine kinase and Ser/Thr kinases. Biochemical data amassed over the past decade established that this site (Thr^{500} in PKC-βII) is regulated by a heterologous kinase; however, the identification of this upstream kinase eluded detection until recently. The discovery in 1997 of PDK-1 as the upstream kinase for the activation loop of the related Akt/protein kinase B (see Ref. 20 for additional details) begged the question as to whether PDK-1 could also be the upstream kinase for the PKCs. In 1998, three laboratories (11, 20, 36) reported that, indeed, PDK-1 phosphorylated the activation loops of conventional, novel, and atypical PKCs. PDK-1 has now been shown to play a pivotal role in cell signaling by phosphorylating the activation loop of diverse members of the AGC family of kinases, including p70S6 kinase, PKN/PRK, p90-Rsk, and serum glucocorticoid-dependent kinase in addition to the PKC isozymes and Akt.

Biochemical and cell biological studies have revealed that phosphorylation by PDK-1 triggers the rapid incorporation of phosphate at two positions on the car-

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[3] Presented by A. C. Newton.

boxy terminus: a turn motif conserved among all PKCs (Thr[641] in PKC-βII) and a hydrophobic phosphorylation motif (Ser[660] in PKC-βII) conserved in conventional and novel PKCs. This motif is present in atypical PKCs except that an acidic residue, Glu, is present instead of the phosphorylatable residue. The hydrophobic motif has attracted considerable attention because it is found in a number of other kinases, notably Akt and S6 kinase, and phosphorylation at this site appears to be tightly coupled to kinase activation. Because phosphorylation of this site in Akt, like that of the activation loop, is serum sensitive, extensive efforts have been devoted to identifying a potential mitogen-sensitive kinase, tentatively referred to as PDK-2.

The PDK-2 site is regulated by intramolecular autophosphorylation. Here we show that there is no PDK-2 for the conventional PKCs. Rather, intramolecular autophosphorylation, triggered by the phosphorylation of the activation loop by PDK-1, regulates the hydrophobic site (6). Autophosphorylation is also shown to be the regulatory mechanism for the hydrophobic site on Akt (66). Thus there is only one upstream kinase for the PKCs, PDK-1.

PKC is activated by engaging its two-membrane-targeting modules on the membrane. PKC is activated by generation of diacylglycerol, which recruits PKC to the membrane. Membrane binding is achieved by two separate membrane-binding modules, the C1 and C2 domains. The former domain binds diacylglycerol (or phorbol esters) and phosphatidylserine, and the latter binds anionic phospholipids in a Ca^{2+}-dependent manner. The binding energy resulting from engaging these domains on the membrane contributes to release of the pseudosubstrate from the substrate-binding cavity. This stretch of sequence occupies the substrate-binding cavity when PKC is inactive and is expelled from the site on activation.

In summary, PKC is under the coordinated regulation of a protein kinase linked to the phosphoinositide 3-kinase signaling pathway and by diacylglycerol. Understanding how PDK-1 is regulated is central to understanding the cellular regulation of PKC. In addition to the phosphorylation discussed here, the activity of PKC isozymes is fine-tuned by tyrosine phosphorylation and, in some cases, induced by oxidative stress as well as isozyme-specific Ser/Thr phosphorylation. Thus phosphorylation presents a new facet in our understanding of the intricate regulation of PKC family members.

OPPOSING ROLE FOR PKC ISOZYMES IN PROTECTION FROM ISCHEMIC INJURY[4]

PKC isozymes are homologous enzymes in which the functional specificity is determined by their subcellular localization (Fig. 3). After activation, each isozyme is translocated to a unique subcellular site where it is anchored by specific proteins, collectively termed RACKs (45). In the past few years, Souroujon and
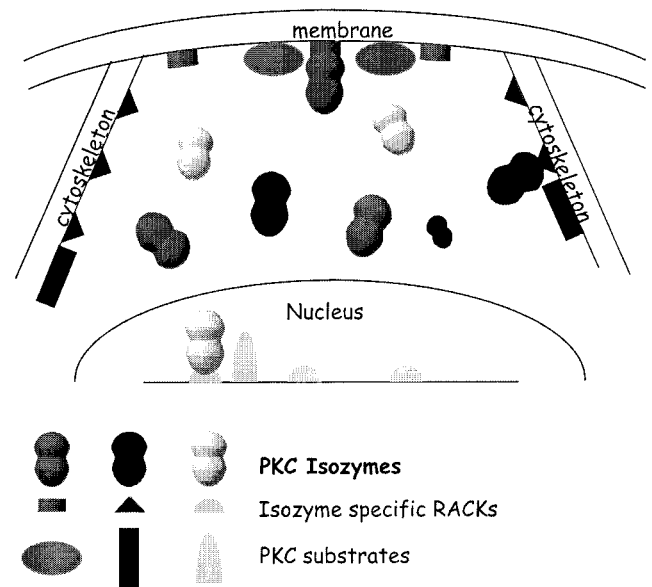
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[4] Presented by D. Mochly-Rosen.

Fig. 3. Schematic diagram showing that substrate specificity of PKC is determined by the localization of individual PKC isozymes after activation. Isozyme-specific localization is due to binding of the activated isozymes to their corresponding anchoring proteins, termed receptors for activated C-kinase (RACKs).

Mochly-Rosen (61) have generated isozyme-specific inhibitors that interfere with protein-protein interactions of individual PKC isozymes and their corresponding RACKs. Dorn et al. (18) and Ron and Mochly-Rosen (58) have also generated isozyme-selective agonists of PKC that inhibit intramolecular interaction in PKC. This effect allows activation and binding of individual isozymes with their RACKs. These inhibitors and activators are short peptides that are introduced into cells by a variety of methods, most recently by conjugating them to a cell permeable peptide derived from the Antennapedia protein (e.g., see Ref. 18). Using these tools, we then determined the role of individual PKC isozymes in the response of cardiac myocytes to ischemia.

Previous work (54, 60) demonstrated that protection from ischemic damage can be induced by subjecting the heart to a short period of ischemia immediately before the more prolonged insult. This form of protection, termed preconditioning, was thought to be mediated by activation of PKC. We found that cardiac myocytes contain at least six different PKC isozymes (17) but that preconditioning results in activation of only two isozymes, PKC-δ and PKC-ε (25). With the isozyme-specific inhibitors and activators that we developed, the role of PKC-ε in this process was determined.

Introduction of a PKC-ε-selective inhibitory peptide, εV1-2, into neonatal cardiac myocytes prevented their protection that is induced by preconditioning (25). In contrast, inhibitors of other isozymes did not prevent cardioprotection by preconditioning (25). These data indicate that PKC-ε activation is required for the protective effect induced by preconditioning. To determine whether activation of PKC-ε is sufficient to produce protection from ischemia-induced cell death, we used
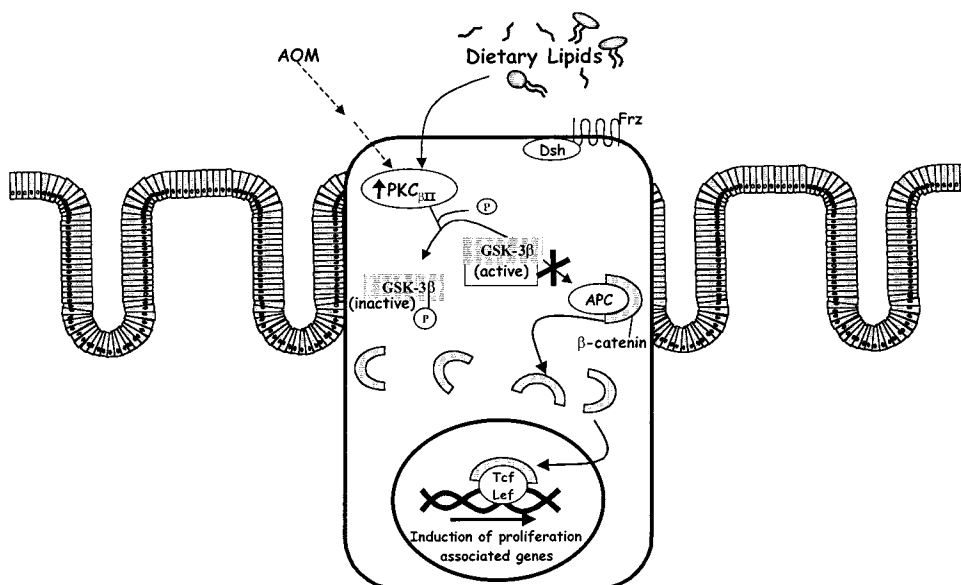
Fig. 4. The role of PKC-βII in colonic epithelial hyperproliferation and carcinogenesis induced by the chemical azoxymethane (AOM). Schematic diagram demonstrates the role of PKC-βII in the Wnt signaling cascade. Frz, frizzled protein; Dsh, disheveled protein; GSK-3β, glycogen synthase kinase-3β; P, phosphorylation; APC, adenomatous polyposis coli protein; Tcf, T-cell factor; Lef, lymphocyte enhancer-binding factor. (Modified from Ref. 47.)

the PKC-ε-selective translocation agonist ΨεRACK. Introduction of ~5 nM of this eight-amino acid peptide resulted in ~70% reduction in ischemia-induced cell death of neonatal and freshly isolated adult cardiac myocytes (18). The protection induced by ΨεRACK was inhibited by the PKC-ε-selective antagonist εV1-2 as well as by inhibitors of the catalytic activity of PKC (18). We then introduced ΨεRACK as a transgene in mouse hearts under the regulation of α-myosin heavy chain, which is expressed selectively in the heart and only after birth. After prolonged no-flow ischemia, there was a faster functional recovery of the hearts from the ΨεRACK transgenic mice compared with that from nontransgenic littermates (18). Moreover, >60% reduction in the amount of cardiac damage, determined by release of a cardiac-specific cytosolic enzyme, creatine phosphokinase, was observed (18). Together, these data indicate that activation of PKC-ε is required and sufficient to produce protection from ischemic damage in isolated cells and in transgenic mice.

Recent work with a PKC-δ-selective agonist and antagonist demonstrated that PKC-δ mediates damage induced by ischemia. Therefore, opposing effects of individual PKC isozymes, unmasked by using isozyme-selective tools, can be induced by the same cell stimulus. Our data also suggest that a PKC-ε-selective agonist will produce a better cardiac protection than isozyme nonselective agonists of PKC. Future studies will determine whether ΨεRACK or compounds that mimic ΨεRACK could be used as therapeutics in treating ischemic heart disease in humans.

IMPORTANCE OF PKC ISOZYMES IN CELL PROLIFERATION[5]

PKC has been implicated in the control of cellular proliferation, differentiation, and survival in many tissue types including the colonic epithelium (49). Indirect evidence also suggests a role for PKC activity in colon carcinogenesis. However, the specific role of individual PKC isozymes in this process has not been directly assessed. Here we report on the role of PKC-βII in colonic epithelial cell proliferation and progression to colon carcinogenesis. We have analyzed the pattern of expression of PKC isozymes during the process of colon carcinogenesis in vivo using a mouse carcinogen model. Immunoblot and quantitative RT-PCR analysis were used to compare protein and mRNA levels for PKC isozymes in normal mouse colonic epithelium, aberrant crypt foci (ACF; preneoplastic lesions of the colon), and colon carcinomas. A dramatic increase in PKC-βII protein was observed in both ACF and colon tumors relative to normal colonic epithelium. In contrast, PKC-α and PKC-βI (a splicing variant of PKC-βII) protein was slightly decreased in ACF and dramatically reduced in colon tumors relative to normal colonic epithelium. Quantitative RT-PCR analysis revealed that PKC mRNA levels did not correlate with PKC protein levels, indicating that expression of PKC isozymes is likely regulated at both the transcriptional and translational/posttranslational levels. Hocevar et al. (30) and Murray et al. (46) have demonstrated that PKC-βII is required for cellular proliferation of human leukemia cells in culture. To investigate PKC-βII function in the colonic epithelium in vivo, Murray et al. (47) generated transgenic mice that express elevated PKC-βII in the intestinal epithelium. Transgenic PKC-βII mice exhibit hyperproliferation of the colonic epithelium and an increased susceptibility to azoxymethane-induced ACF and colon tumor formation (47). Furthermore, transgenic PKC-βII mice exhibit elevated colonic β-catenin levels and decreased glycogen synthase kinase-3β activity, indicating that PKC-βII stimulated the Wnt-adenomatous polyposis coli (APC)-β-catenin proliferative signaling pathway in vivo (Fig. 4).

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[5] Presented by A. P. Fields.

Our results demonstrate that specific, reproducible changes in PKC isozyme expression occur during colon carcinogenesis and that PKC isozyme expression patterns are controlled by a combination of transcriptional and nontranscriptional mechanisms. Similar changes in PKC isozyme expression were observed in human colon tumors, demonstrating the relevance of these findings to human disease. Elevated expression of PKC-βII in transgenic mice led to hyperproliferation of the colonic epithelium and increased susceptibility to colon carcinogenesis. Taken together, these data demonstrate that elevated PKC-βII is an early event in colon carcinogenesis that plays a direct promotive role in colonic epithelial cell proliferation and colon carcinogenesis, possibly through activation of the APC-β-catenin signaling pathway.

IMPORTANCE OF PKC ISOZYMES IN APOPTOSIS[6]

Apoptosis is a genetically programmed form of cell death that is important in development and for the removal of tumor cells and cells injured by chemicals and radiation. Apoptosis can be initiated via cell surface death receptors such as Fas and tumor necrosis factor-α or by agents that cause cell damage. Chemicals and irradiation induce apoptosis via a mitochondrial-dependent pathway (Fig. 5). Specific changes in the mitochondrial membrane result in the release of cytochrome c, the subsequent activation of caspase-9, and activation of effector caspases such as caspase-3, -6, and -7 (63). Activated effector caspases dismantle the cell through cleavage of cell proteins, resulting ultimately in DNA fragmentation and cell death. The Bcl-2 family of proteins plays a major role in regulating the mitochondrial events, with proteins such as Bcl-2 and Bcl-xL suppressing death, whereas Bax, Bad, and other proteins induce cell death (35).

PKC plays a fundamental role in the regulation of cell proliferation and differentiation, and recent studies (5, 7, 48, 68) suggest that it is also involved in the regulation of cell survival. Early approaches to defining the role of PKC in apoptosis relied on activation of PKC by phorbol 12-myristate 13-acetate or inhibition by pharmacological agents. This work (41) showed that activation of PKC may be either proapoptotic or antiapoptotic depending on the cell type. More recently, studies (5, 7, 22, 48, 56, 68) have begun to define isoform-specific functions of PKC in the apoptotic pathway. PKC isoforms that appear to be antiapoptotic include PKC-α, PKC-βII, and PKC-ε and the atypical isoforms PKC-λ/ι and PKC-ζ. For example, expression of a dominant negative form of PKC-α induces apoptosis in COS-1 cells (68) and in salivary gland epithelial cells (Matassa A and Reyland ME, unpublished data), suggesting that PKC-α may be a survival factor (Fig. 5). Likewise, overexpression of PKC-βII protects small cell lung cancer cells against c-myc-induced apoptosis (5). The atypical PKC isoforms PKC-λ/ι and PKC-ζ, are downstream effectors of phosphoinositide 3-kinase and
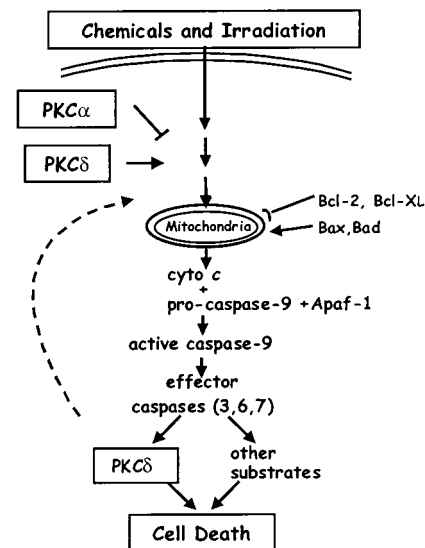

Fig. 5. The role of PKC isozymes in chemical and radiation-induced apoptosis. A diverse group of agents, including DNA-damaging agents and cell toxins, induce a mitochondrial-dependent apoptotic pathway. Release of cytochrome (cyto) c from the mitochondria and binding to apoptotic protease activating factor-1 (Apaf-1) result in activation of caspase-9, which, in turn, activates downstream effector caspases. Inhibition of PKC-α can activate, whereas inhibition of PKC-δ can suppress, this pathway. In addition, cleavage and activation of PKC-δ by caspase may serve to amplify specific events in the apoptotic pathway.

are required for mitogenic activation in oocytes and fibroblasts, suggesting that they may also transduce a survival signal. In agreement with this, Murray and Fields (48) showed that PKC-λ/ι protects human K562 leukemia cells from apoptosis. Berra et al. (7) have shown that exposure of cells to apoptotic stimuli such as ultraviolet radiation leads to a dramatic decrease in the activity of the atypical PKC isoforms PKC-ζ and/or PKC-λ/ι.

In contrast to the antiapoptotic isoforms, PKC-δ is emerging as a common intermediate in the apoptotic pathway induced by chemicals and irradiation (Fig. 5). Proteolytic activation of PKC-δ by caspases releases a catalytically active fragment in cells induced to undergo apoptosis by DNA-damaging agents (22). Activation of PKC-δ by caspase cleavage may serve to amplify downstream events in the apoptotic pathway because expression of the catalytic fragment alone is sufficient to induce caspase activation and apoptosis in a variety of cell types. However, studies from our laboratory (56) indicate that PKC-δ activity is also required for apoptosis at a point upstream of caspase activation. Inhibition of PKC-δ with rottlerin or by expression of a dominant negative PKC-δ protein suppresses caspase activation and DNA fragmentation in salivary gland epithelial cells, indicating that PKC-δ is required for these events (56). Furthermore, expression of a dominant negative mutant of PKC-δ is sufficient to inhibit phorbol ester-induced apoptosis in prostate cancer cells, which does not result in the caspase-directed cleavage and activation of PKC-δ (23). Thus PKC-δ may function at two or more points in the apoptotic

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[6]Presented by M. E. Reyland.

pathway. These functions of PKC-δ may be distinct, wherein activated full-length PKC-δ plays a role in the initiation of apoptosis and the cleavage and activation of PKC-δ by caspase result in the amplification of apoptosis.

The identification of both pro- and antiapoptotic isoforms suggests that PKC may function as a molecular sensor, promoting cell survival under favorable conditions and executing the death of abnormal or damaged cells.

LESSONS FROM PKC KNOCKOUT MICE[7]

Of the 12 PKC isozymes identified thus far, PKC-γ, -β, -ε, and -θ have been mutated to generate null mice by homologous recombination. Neural, immunologic, and endocrine phenotypes have been reported. PKC-γ-null mice were the first to be generated by Abeliovich et al. (2). Because this isozyme is exclusively expressed in the central nervous system, all phenotypes involved central nervous system function. The first one described was a deficit in spatial learning observed during a Morris water maze probe test (2). The deficit was mild and could be overcome by intensive training. It was associated with impaired contextual fear conditioning. Both findings suggest hippocampal dysfunction and were associated with impaired long-term potentiation in CA1 hippocampal pyramidal neurons after high-frequency stimulation of CA3 axons (2). A subsequent study by the same laboratory disclosed a second phenotype of gait ataxia associated with persistent multiple climbing fiber synapses on cerebellar Purkinje cells due to impaired synapse elimination during development (10).

Other laboratories have identified additional phenotypes in PKC-γ mice. Malmberg et al. (42) found that these mice showed normal pain responses but decreased hyperalgesia after mechanical or inflammatory peripheral nerve injury. Martin et al. (43) observed that PKC-γ is expressed by a subset of neurons in lamina II of the dorsal spinal cord and is induced by chronic inflammation. Furthermore, nerve injury increases the levels of neuropeptide Y and neurokinin-1 receptor immunoreactivity and decreases substance P receptor immunoreactivity in the dorsal horn of the spinal cord, but these responses are diminished in PKC-γ-null mice. Taken together, these findings suggest that PKC-γ is important in the neural plasticity within the spinal cord after nerve injury that contributes to neuropathic pain.

In a different set of studies, Harris et al. (28) found that PKC-γ-null mice show a decreased sensitivity to ethanol-induced hypothermia and to the sedative effects of ethanol as measured by the duration of drug-induced loss of righting reflex (LORR). In a subsequent study, these mice failed to develop tolerance to ethanol-induced LORR (9). The decreased sensitivity to ethanol was lost when the mice were backcrossed onto a C57BL/6J background but could be recovered by breeding the C57BL/6J mice with 129SvEvTac mice. These findings indicate the polygenic nature of ethanol responses and demonstrate the need to control for effects of genetic background in studies with null mice.

PKC-β mice were initially found to demonstrate deficiencies in B-cell function and impaired humoral immune responses (37). More recent work (50) has documented deficits in mast cell degranulation and interleukin-6 production. It is not yet certain if these changes are due to developmental effects of the null mutation or loss of the isozyme in adult tissues. Recently, PKC-β-null mice have been found to show a modest increase in insulin-stimulated translocation of GLUT-4 glucose transporters and in glucose transport in some tissues (62). This can be rescued in part by transgenic expression of PKC-βI, suggesting that it is due to the loss of that isozyme. PKC-θ-null mice were recently reported to show striking deficits in adult T-cell signaling, particularly in T-cell receptor-initiated activation of nuclear factor-κB (64). This deficit was not evident in thymocytes, suggesting that it resulted from a loss of the isozyme in adult T cells. These findings suggest that it may be possible to develop inhibitors of PKC-θ that could be used as immunosuppressants.

My laboratory recently generated PKC-ε-null mice. Although these mice display normal responses to noxious thermal and mechanical stimuli, they show decreased nociceptor sensitization after local injection of epinephrine (33). Similar findings were obtained in rats injected locally with a specific peptide inhibitor of PKC-ε, confirming that responses observed in null mice are due to the loss of PKC-ε function in mature neurons. This inhibitor also inhibited epinephrine-induced enhancement of tetrodotoxin-resistant sodium current in rat dorsal root ganglion neurons. When injected locally, the inhibitor also decreased carrageenan-induced hyperalgesia in rats. These findings suggest that PKC-ε inhibitors may prove useful in the treatment of pain states. In another study, our laboratory (31) found that PKC-ε-null mice are supersensitive to the sedative effects of ethanol, barbiturates, and benzodiazepines and that this correlates with enhanced sensitivity of γ-aminobutyric acid$_A$ receptors to the agonist properties of these drugs in vitro. This increase in sensitivity to ethanol was associated with decreased voluntary alcohol consumption. This supports other findings in rats and humans, indicating an inverse correlation between alcohol consumption and sensitivity to acute alcohol intoxication. These studies suggest that inhibitors of PKC-ε may reduce alcohol consumption and prove useful in the treatment of alcoholism.

FUTURE DIRECTIONS[8]

The advances in PKC signaling described here reveal at least some of the novel experimental approaches now available for investigation of this remarkably complex enzyme family. They also underscore the importance of

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[7] Presented by R. O. Messing.

[8] Presented by E. C. Dempsey.

PKC in lung health and disease and the feasibility of selecting PKC isozymes as therapeutic targets. As this area of signaling has matured, it also provides insights into how best to study multiple isozymes in other signal transduction cascades. Immediate challenges in the pulmonary field include *1*) finding more efficient and less toxic ways to transiently apply isozyme-specific antagonist strategies to more biologically relevant primary cultures of epithelial, endothelial, smooth muscle, and fibroblast cells that are known to be heterogeneous and to rapidly change in culture; *2*) more carefully validating the effects of putative PKC antagonists used in isolated lung preparations and whole animal models; and *3*) developing strategies for targeting gene mutations to additional types of cells in the lung. Other compelling questions raised and areas for future investigation are the following:

1) How is the activity and expression of PDK-1 regulated in lung cells? Are there developmental or differentiation-associated differences in activity or expression of PDK-1? Is there heterogeneity in activity or expression of PDK-1 among different subtypes of lung cells? What effect do relevant forms of cellular stress like hypoxia, hyperoxia, shear stress, and cigarette smoke have on PDK-1 function?

2) Are there PKC isozyme-specific differences in susceptibility to H_2O_2-induced tyrosine phosphorylation in lung cells? What is the biological importance of this form of activation in the lung?

3) What regulates expression of PKC binding proteins (i.e., including RACKs and caveolins) in lung cells? Is their expression dependent on developmental stage, state of differentiation, or subpopulation? Is their expression altered by relevant forms of cellular stress? If so, by what mechanism? What are the structural determinants of their interaction with and regulation of PKC isozymes?

4) In a given intracellular milieu, what are the primary determinants of function for a PKC isozyme? And how can a change in cell type or shift in cell phenotype after injury lead to a change in the function of a PKC isozyme?

5) What factors regulate the balance between expression, phosphorylation, and degradation of individual isozymes in lung cells? Do individual isozymes contribute to the regulation of their own expression and/or the expression of other isozymes? Do changes in levels of individual isozymes occur under conditions of cellular stress and do they contribute to change in phenotype? And by what mechanism?

6) What effect does targeted disruption of relevant PKC isozyme genes have on susceptibility to cellular stress or injury in vivo? Do some isozymes serve a protective role? Do others increase susceptibility or extent of injury?

7) Can pharmacological and molecular approaches to inhibit or activate selected PKC isozymes attenuate or reverse pathological conditions like pulmonary edema, lung injury, asthma, and pulmonary hypertension in vivo?

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