

Neuroprotective versus tumorigenic protein kinase C activators

T.J. Nelson and D.L. Alkon

Blanchette Rockefeller Neurosciences Institute, West Virginia University School of Medicine, Morgantown, WV 26506, USA

Protein kinase C (PKC) activators possess potent neurotrophic and neuroprotective activity, thus indicating potential applications in treating neurodegenerative diseases, stroke and traumatic brain injury. Although some activators, such as bryostatin and gni-dimacrin, have been tested as antitumor agents, others, such as phorbol esters, are potent tumor promoters. All PKC activators downregulate PKC at high concentrations and long application times. However, tumorigenic activators downregulate certain PKC isozymes, especially PKC δ , more strongly. Tumorigenic activators possess unique structural features that could account for this difference. At concentrations that minimize PKC downregulation, PKC activators can improve long-term memory, reduce β -amyloid levels, induce synaptogenesis, promote neuronal repair and inhibit cell proliferation. Intermittent, low concentrations of structurally specific, non-tumorigenic PKC activators, therefore, could offer therapeutic benefit for a variety of neurologic disorders.

Biphasic effects of PKC activation

Although protein kinase C (PKC) is found in species from yeast to humans, its importance in cellular signaling has focused much attention on its role in neurons and neurological disorders. Recently, researchers have demonstrated that PKC activators have remarkable neuroprotective effects in animals that could prove beneficial in the treatment of acute and long-term neurodegenerative diseases. They can induce synaptic maturation, inhibit apoptosis, increase neurotrophin levels and reduce β -amyloid (A β ; see Glossary) levels [1,2]. Moreover, they can counteract multi-drug resistance, strengthen the immune system and inhibit cancer-cell proliferation [3]. However, PKC activators can also induce apoptosis, increase A β levels, produce inflammation and act as powerful tumor promoters. In fact, for nearly every beneficial effect of PKC activation, it is possible to find a cell type, a time point or a PKC isoform where a PKC activator produces effects precisely the opposite of what is intended.

This seemingly contradictory behavior results mainly from the biphasic nature of PKC activation. In a process known as downregulation, PKC that has been activated and bound to the membrane (in contrast to non-activated PKC, which remains in the cytosol) is rapidly and preferentially degraded [4]. Thus, all PKC activators also cause PKC downregulation. When downregulation occurs, PKC activators can cause undesirable side effects instead of

providing neuroprotection. At the same time, unless the time-dependent effects on each PKC isozyme are understood, useful drug candidates could be prematurely dismissed. To avoid these problems, we need a better understanding of the biochemistry of PKC activation. Here, we discuss the current understanding of the basic biochemistry of PKC activators and the structural features that distinguish tumor-promoting and neuroprotective PKC activators.

Mechanism of PKC activation

PKC activation consists of three steps: phosphorylation, binding to signaling lipids and translocation from the cytosol to the membrane (Figure 1). Cytosolic PKC is normally inactive because its pseudosubstrate region, located on the PKC C1 domain, loops back and binds to the substrate-binding catalytic region located on the C4 domain, thereby preventing PKC from binding to any substrate proteins. The first step in activating PKC is

Glossary

Atypical PKC isoforms (ζ and ι/λ): phospholipid-dependent PKC isoforms; they do not rely on calcium or DAG.

β -amyloid: toxic 39 to 43 amino acid peptide (also known as A β) produced by cleavage of APP by β - and γ -secretases. A β is the main constituent of amyloid plaques and is the leading candidate for the causative agent in Alzheimer's disease.

C1 domain: a 'constant' region in PKC that binds DAG. Conventional and novel isoforms possess two non-identical C1 regions, called C1a and C1b, which are cysteine-rich, zinc-binding motifs. The pseudosubstrate motif is immediately adjacent to the C1 domain on the N-terminal side. The C1 domain is present but inactive in atypical PKC isoforms.

C2 domain: a region in PKC that binds calcium and phosphatidylserine (PS). The C1 and C2 domains together constitute the PKC regulatory domain. Active PKC binds the membrane via the C2 domain. Only the conventional PKC isoforms possess a functional calcium sensor. Novel and atypical PKC isoforms lack a C2 domain.

C3 domain: the PKC ATP-binding region.

C4 domain: the substrate-binding catalytic region of PKC. The C3 and C4 domains together comprise the catalytic domain. C3 and C4 domains are present in all PKC isoforms.

Cdk2: cyclin-dependent kinase 2, a kinase that regulates the cell cycle. Cdk2 interacts with cyclins A, B and E and is essential for the G1 to S transition.

Conventional PKC isoforms (α , β , β II and γ): PKC isoforms that are dependent on calcium, DAG and phospholipid.

Neurotrophin: a family of proteins that promote neuronal survival. Neurotrophins include brain-derived neurotrophic factor, nerve growth factor and neurotrophins 3 and 4.

Novel PKC isoforms (δ , ϵ , η and θ): PKC isoforms that are dependent on DAG and phospholipid; not dependent on calcium.

PDK1: 3-phosphoinositide-dependent protein kinase-1, a kinase responsible for phosphorylating a variety of other kinases including PKC, AKT and S6K.

PKM: a proteolyzed form of PKC containing only the catalytic domain.

α -Secretase: a family of proteases that cleave amyloid precursor protein (APP) to produce sAPP α (soluble APP α). α -Secretases include TACE (tumor necrosis factor- α converting enzyme) and ADAM10 (a disintegrin and metalloprotease domain protein 10).

Corresponding author: Nelson, T.J. (tjnelson@brneurosci.org).

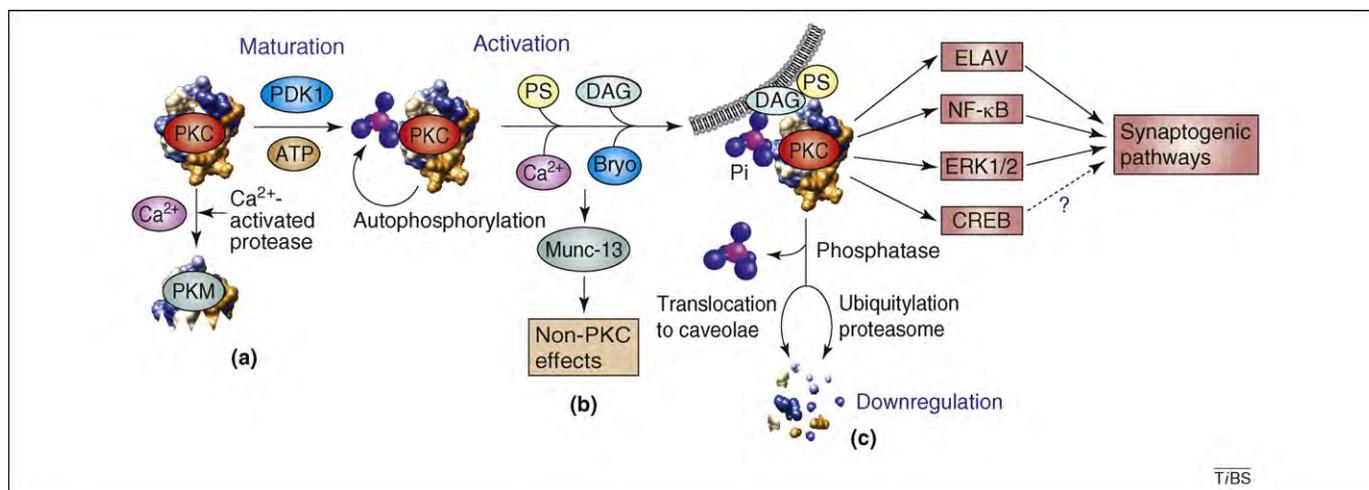


Figure 1. PKC regulation and transcription pathways for synaptogenesis. PKC (orange) undergoes a well-defined sequence of maturation (a), activation (b) and downregulation (c). In the maturation phase (a), PKC is phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1, blue); this is followed by autophosphorylation. These steps confer catalytic competence to PKC. (b) Conventional and novel PKC isoforms bind to phosphatidylserine (PS; yellow) and DAG (green), which cause activation and translocation to the plasma membrane, Golgi membrane or other compartments. Conventional PKC isoforms also bind to calcium (pink), which participates in PS binding. Activated PKC participates in a variety of biochemical pathways, including activation of ELAV mRNA-stabilizing proteins, nuclear factor κ B (NF- κ B), α -secretases and CREB (cAMP-response-element-binding proteins). Activation of these pathways could promote synaptogenesis. (c) Gradually, PKC is dephosphorylated by various phosphatases and can then be degraded by the proteasome or by proteases in the caveolae. PKC can also be degraded by Ca^{2+} -activated proteases, producing PKM. DAG and PKC activators such as bryostatin (Bryo) can also bind other DAG-binding proteins, including Munc-13 (Box 1), which is involved in synaptic vesicle exocytosis. The '?' denotes that it is hypothetical.

phosphorylation or 'maturation'. Conventional (α , β I, β II and γ) and novel (δ , ϵ , η and θ) PKC isoforms can be phosphorylated on three separate regions: the activation loop, the turn motif and the hydrophobic motif. Maturation entails phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) at a threonine on the activation loop of PKC. This phosphorylation step is essential for formation of the catalytically competent conformation of PKC [5,6]. PKC can then be autophosphorylated at a serine in the hydrophobic motif and a threonine in the turn motif; this stabilizes the active conformation, resulting in activation.

Binding of 1,2-diacyl-sn-glycero-3-phospho-L-serine (phosphatidyl-L-serine, PS) and 1,2-diacylglycerol (DAG) causes PKC to translocate from the cytosol to the membrane. Membrane binding releases the pseudosubstrate domain from the substrate-binding domain, enabling PKC to become active. PKC membrane binding is stabilized by specific anchoring proteins including receptor for activated C-kinase (RACK) [7]. Some anchoring proteins are isozyme

Box 1. Non-PKC neuroprotective effects of PKC activators

In addition to PKC, DAG-binding PKC activators also bind and activate several other proteins, including chimerins, RasGRPs (exchange factors for Ras), Munc-13 (a scaffolding protein involved in nerve terminal exocytosis), c-Raf (part of the Ras-Raf-MAP kinase pathway), protein kinase D and DAG kinases (which convert DAG to phosphatidic acid). These proteins all possess cysteine-rich C1 domains similar to those found in PKC. Many of these proteins have functions that are complementary to PKC and could be important for neuroprotection. For example, Munc-13 proteins are C1-domain-containing proteins that are involved in synaptic vesicle exocytosis. This finding could help to explain several phenomena observed after bryostatin treatment in animals, such as increases in the numbers of presynaptic vesicles. Bryostatin does not increase the number of presynaptic boutons but, rather, increases the number of mushroom spines associated with individual synaptic boutons [1].

specific and can modulate the isozyme and subcellular specificity of PKC signaling [7].

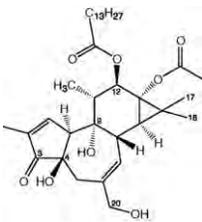
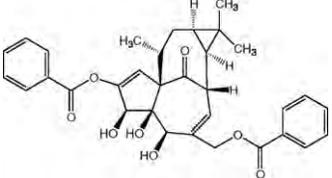
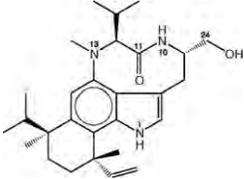
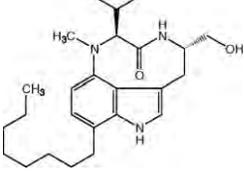
In atypical PKC isoforms (ζ and ν/λ), the serine at the phosphorylation site on the hydrophobic motif is replaced by a glutamate, rendering that phosphorylation site constitutively active. The activation loop also exhibits isoform-specific differences in phosphorylation. For example, the novel isoform PKC δ does not require phosphorylation of threonine in the activation loop for activity. Indeed, PKC δ is unique because its activity is regulated by tyrosine phosphorylation, which is carried out by tyrosine kinases such as Src [8].

Downregulation

Activated, phosphorylated PKC is gradually dephosphorylated by protein phosphatases including the Ser/Thr-specific phosphatase PHLPP (pleckstrin homology domain leucine-rich repeat protein phosphatase), protein phosphatase 1 and protein phosphatase 2A. After dephosphorylation, PKC is downregulated by caveolin-mediated translocation to endosomes followed by ubiquitylation and proteasomal degradation [4]. In neurons, the net effect is to produce a brief pulse of PKC activity in response to a neurotransmitter stimulus, thus preventing continuous PKC activation. Rapid degradation of DAG further shortens the duration of PKC activation.

Another degradation pathway involves caveolae-dependent trafficking of the active enzyme and subsequent proteasome-independent degradation. A third pathway, occurring after bryostatin-mediated activation, involves ubiquitylation of fully phosphorylated PKC and downregulation by the proteasome [9]. Thus, the degradation pathway taken by PKC depends, in part, on how PKC was activated. Some PKC activators, such as phorbol 12-myristate 13-acetate (PMA), induce PKC downregulation primarily through the proteasome pathway [9]. Others,

Table 1. Tumor-promoting PKC activators

Activator	Structure
<p>Phorbol esters</p> <p>Phorbol esters [56] are tetracyclic diterpenoids that mimic DAG. They are natural products obtained from croton oil, which is found in the seeds of several plants including <i>Croton tiglium</i> and several species of <i>Euphorbia</i>. Phorbol esters are the most potent tumor promoters known. In the presence of low doses of carcinogens such as benzo[a]pyrene, nanomolar concentrations of phorbol esters efficiently promote tumor growth.</p>	
<p>Ingenol</p> <p>Ingenol [67] is a diterpenoid related to phorbol, but it possesses much less toxicity. It is derived from the milkweed plant <i>Euphorbia peplus</i>. Although ingenol-3-angelate possesses antitumor activity when used topically, ingenol esters are considered to be highly active tumor promoters [68]. They are regarded as skin irritants, but their systemic toxicity is unknown.</p>	
<p>Teleocidin</p> <p>Teleocidin and aplysiatxin are potent tumor promoters with activity similar to phorbol esters [69]. Teleocidin was named for its ability to kill the teleost fish <i>Oryzias latipes</i>. Aplysiatoxin, a cyanotoxin produced by the blue-green alga <i>Lyngbya majuscula</i>, is unusual in that it contains bromine.</p>	
<p>Octylindolactam V</p> <p>Octylindolactam V is a lactam-type alkaloid related to the tumor promoter teleocidin. Octylindolactam and indolactam are 'complete' tumor promoters (compounds that possess both tumor-initiating and tumor-promoting activity). A large number (10–20) of similar benzolactam derivatives have been synthesized [50]; some show selectivity for PKCϵ and PKCθ [70].</p>	

such as bryostatin, use both proteasome-dependent and proteasome-independent pathways. The pathway also depends on the isozyme: PKC α and PKC ϵ require dephosphorylation, whereas PKC δ does not [10]. After activation by phorbol esters, PKC levels in cultured cells rapidly decline and remain well below normal for up to 24 hours. Downregulation by bryostatin is milder, but lasts approximately the same length of time.

Because PKC activation invariably produces some downregulation, it is essential to measure PKC activity in any study of PKC activators; otherwise, effects attributed to PKC activators might actually represent downregulation. A useful control is to compare the effects of PKC activators to PKC inhibitors: if PKC activators and inhibitors produce the same effect, then the effect probably represents downregulation and not PKC activation. Unfortunately, few experiments have so far included these controls.

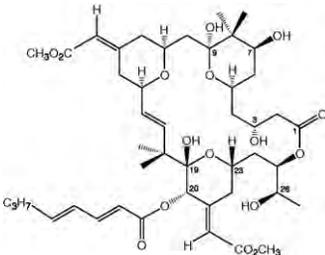
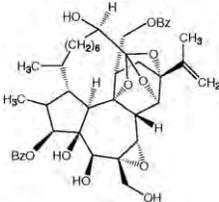
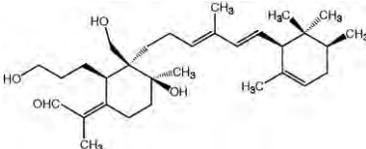
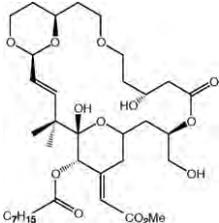
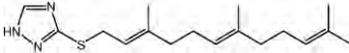
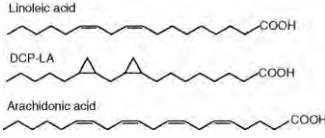
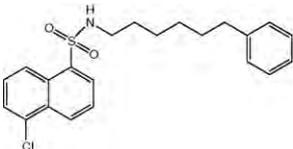
DAG-binding site

Many PKC activators, including bryostatin, phorbol esters and other macrocyclic lactams and terpenes (Tables 1 and 2), act by binding to the DAG-binding site in the PKC C1 domain (Figure 2a). Both bryostatin and phorbol esters bind with sufficiently high affinity that the kinetic parameters approach those seen with irreversible suicide inhibitors. Owing to the low rate of dissociation, biological effects of PKC activation can continue long after free bryostatin is no longer detectable in the blood.

On the basis of X-ray crystallography results and modeling studies [11–13], it is possible to construct a detailed picture of the interaction of bryostatin with PKC (Figure 2a). The oxygen at position 1 of bryostatin (corresponding to position 4 of phorbol ester) is bound by hydrogen bonds from the main-chain carbonyl of Gly253, and the alcohol at position 26 (corresponding to position 20 of phorbol) is bound by three hydrogen bonds from the amide of Thr242 and the main-chain carbonyl of Thr242 and Leu251. Phorbol esters form a fifth hydrogen bond between the main-chain amide of Gly253 and C3 domain of phorbol [11]. Because bryostatin lacks a heteroatom corresponding to the C3 ketone on phorbol [13], bryostatin would be linked to PKC by only four instead of the five hydrogen bonds found for phorbol esters. The bryostatin aliphatic tail, like that in the phorbol esters, is inserted in the lipid bilayer and anchors PKC to the membrane.

Although the differences in binding between bryostatin and phorbol esters are small, they produce marked differences in binding among the PKC isozymes. PKC δ downregulation by bryostatin is only transient [14], whereas downregulation by phorbol esters can take several days to recover [4,15]. This could, in part, be caused by unique properties of the PKC δ C1 domain [15]. Conventional and novel forms of PKC possess two C1 domains called C1a and C1b [16]. The relative affinities of C1a and C1b for PKC activators differ among PKC isoforms. For example, phor-

Table 2. Non-tumor-promoting PKC activators

Activator	Structure
<p>Bryostatin</p> <p>Bryostatin is a macrolide lactone produced by a bacterial symbiont of the bryozoan <i>Bugula neritina</i>. Bryostatins compete for the PKC DAG-binding site with very high affinity (≈ 1.35 nM) producing a brief activation period followed by a prolonged downregulation.</p>	
<p>Gnidimacrin</p> <p>Gnidimacrin is a daphnane-type diterpene that displays potent antitumor activity at concentrations of 0.1–1 nM. It acts as a PKC activator at a concentration of ≈ 3 nM. Gnidimacrin arrests the cell cycle in the G1 phase in a PKC-dependent manner [71].</p>	
<p>Iripallidal</p> <p>Iripallidal is a bicyclic triterpenoid from <i>Iris pallida</i> that displays anti-proliferative activity. It binds PKCα with high affinity ($K_i = 75.6$ nM). It induces ERK1/2 phosphorylation in a RasGRP3-dependent manner [72].</p>	
<p>Bryologues</p> <p>Wender <i>et al.</i> [13] created and studied a variety of bryostatin analogs ('bryologues'). Structurally they are all similar, but they vary greatly in their affinity for PKC (from 0.25 nM to >10 μM).</p>	
<p>Farnesyl thiotriazole</p> <p>Farnesyl thiotriazole (FTT) is a synthetic isoprenoid compound that activates PKC with a K_d of 2.5 μM. FTT is a benign molecule with a potency comparable to DAG [29]. Treatment of neutrophils with FTT causes the release of superoxide radical [73].</p>	
<p>DAG derivatives</p> <p>Only 1,2-sn-diacylglycerols, and not 2,3-sn-diacylglycerols, 1,3-diacylglycerols or triglycerides, activate PKC. Numerous PKC-activating DAG derivatives are known [74]. PKC activation by DAG is transient because DAG is rapidly metabolized by DAG kinase and lipase. DAGs possessing an unsaturated fatty acid are most active [75].</p>	
<p>PUFAs</p> <p>Unsaturated fatty acids, including arachidonic acid and minerval (2-hydroxy-9-cis-octadecenoic acid), are effective PKC activators. The linoleic acid derivative DCP-LA selectively activates PKCϵ [76]. DCP-LA interacts with the PKC PS-binding site. Certain eicosanoid metabolites, including lipoxin A, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid [77], 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) and the isoprenoid retinoic acid, are also reported to activate PKC.</p>	
<p>Naphthalenesulfonamides</p> <p>SC-10 (<i>N</i>-(<i>n</i>-heptyl)-5-chloro-1-naphthalenesulfonamide) and related naphthalenesulfonamides activate PKC in a calcium-dependent manner [78]. Structurally, these compounds are similar to the calmodulin antagonist W-7, but have no effect on Ca²⁺/calmodulin-dependent protein kinase.</p>	
<p>Oxidized lipids</p> <p>The hydroperoxide of 1,2-diacylglycerol (DAG-OOH) is up to seven times more potent than 1,2-diacylglycerol [79]. A stable analog of thromboxane, 9,11-epithio-11,12-methano-thromboxane A2 (STA2), activates PKC at concentrations of 10 nM [80].</p>	

bol esters bind almost exclusively to the PKC δ and PKC θ C1b domains [17,18]. In PKC α , DAG-dependent translocation is mediated primarily by C1a [19], whereas C1b preferentially binds to phorbol esters [20]. By contrast, the

PKC ϵ and PKC γ C1a and C1b domains are involved in phorbol ester binding and translocation [20,21].

Mutation studies indicate that these differences between C1a and C1b domains also affect the interaction

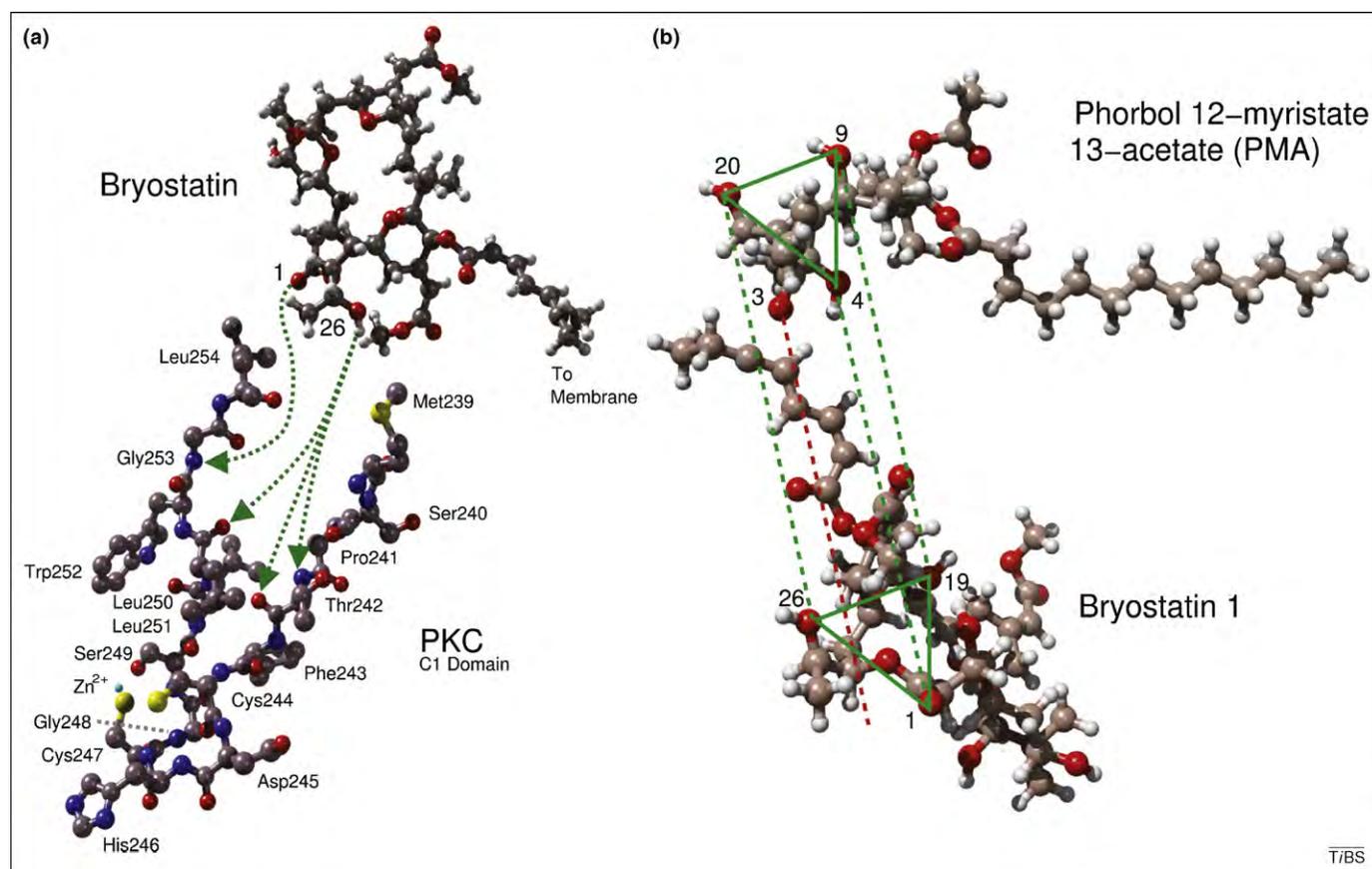


Figure 2. Molecular models of PKC activators. **(a)** Bryostatin bound to PKC. Bryostatin binds to the PKC DAG-binding site, as shown in this molecular model based on the X-ray crystallographic study of Zhang *et al.* [11] and bryostatin modeling studies [12,13]. This model illustrates a small portion of the PKC δ C1 domain. The bryostatin aliphatic tail, like that in the phorbol esters, is inserted in the lipid bilayer and anchors PKC to the membrane. The oxygen at position 1 of bryostatin is bound by hydrogen bonds (green arrows) to the main-chain carbonyl of Gly253. The alcohol at position 26 is bound by three hydrogen bonds to the amide of Thr242 and the main-chain carbonyl of Thr242 and Leu251. Phorbol esters form an additional hydrogen bond to the amide of Gly253. The surrounding regions of the protein (Met239 and Leu254) form a small pocket. Two additional zinc coordination bonds and the PKC hydrogens are not shown (C, brown; O, red; N, blue; S, yellow; H, white; Zn, light blue). **(b)** Comparison of bryostatin and the phorbol ester PMA. Although both molecules possess three oxygens (illustrated by green triangle) in the precise locations needed for binding, their 3D configurations are completely different. Notably, their hydrophobic side chains point in nearly opposite directions. Unlike bryostatin, phorbol esters are nearly planar, with the heteroatoms protruding from the fused ring structure. Phorbol ester also possesses a ketone at position 3, which participates in PKC binding. Corresponding oxygens are present in many tumor-promoting PKC activators, but not in bryostatin, gnidimacrin or DAG. These structural differences might account for the differences in their biological effects. Numbers indicate the corresponding atoms on the carbon backbone. (O, red; C, brown; H, white).

with bryostatin. Although both bryostatin and phorbol esters possess the three oxygen atoms in the precise 3D orientation needed for binding and activation, the other portions of the two molecules are markedly different (Figure 2b). Unlike bryostatin, the phorbol ester is nearly planar, with the oxygen functional groups protruding from the relatively flat ring system. Even the hydrophobic tails are oriented in different directions. These differences could help to explain why the concentration–response curve of PKC δ downregulation by bryostatin is U-shaped, with a maximal effect at 1 nM [15]. Indeed, for bryostatin, less is more: >10 nM there is less net activation than at lower concentrations. This phenomenon is also observed *in vivo*: rather than enhancing memory, excessive bryostatin treatment blocks learning altogether [22].

Differences in the administration schedule can also affect whether PKC activators produce activation or downregulation. In one study, levels of phosphorylated mitogen-activated protein (MAP) kinase, a surrogate marker for PKC activation, were dramatically increased when bryostatin was administered to patients eight times over a period of 14 days [23]. This could represent a rebound effect caused by increased PKC synthesis in response to pro-

longed or repeated downregulation. Another way of avoiding downregulation is by administering extremely low bryostatin concentrations [24].

PS-binding site

Comparatively little is known about the PKC PS-binding site. In conventional PKC isoforms, PS and calcium bind to the PKC C2 domain, which is responsible for its initial membrane binding. DAG binds to PKC after PS docks it to the membrane, which results in membrane penetration and activation. X-ray crystallography showed that the PS phosphate is directly coordinated to one of the three Ca²⁺ ions that bind within the C2 domain of conventional PKCs [25]. The C1 region is also crucial for PS binding; DAG binding to the C1 site increases the PKC specificity for PS. The calcium-binding sites are nonfunctional in the novel PKC isoforms, and PKC residues in the C2 domain bind directly to acidic membrane phospholipids. In these cases, docosahexaenoic acid, retinoic acid and other polyunsaturated fatty acids and derivatives such as 8-[2-(2-pentylcyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) bind to the PS-binding site, producing activation.

Indirect PKC activation

A variety of other growth factors, including fibroblast growth factor (FGF)-18 and insulin-like growth factor (IGF-I), can activate PKC indirectly. FGF-18 expression, brain insulin and insulin growth factor receptors are up-regulated during learning [26,27]. Fibroblast growth factors activate PKC indirectly by phospholipase C γ activation [28]. IGF-I acts by stimulating the Ser/Thr kinase Akt by way of phosphatidylinositol 3-kinase (PI3K). These kinase pathways intersect with PKC signaling pathways at several points. For example, IGF-I stimulates PKC activity, and PKC phosphorylates insulin-receptor-substrate 1. The DAG-binding site of PKC can also be targeted indirectly by activating phospholipases or by increasing the levels of the endogenous ligand (DAG) using DAG kinase inhibitors such as R59022 and R59949 [29]. These compounds offer additional potential means for activating PKC.

Biological effects of PKC activation

Although research in this area is still in the early stages, one exciting potential new role for PKC activators lies in the treatment of Alzheimer's disease (AD). The factor that correlates most strongly with memory loss in AD is neither cell death nor amyloid plaques, but synaptic loss [30,31]. PKC activators promote DNA transcription and increase neurotrophin levels, thereby enhancing synaptogenesis and protecting against neuronal cell loss. Although the mechanism presumably involves PKC activation, the details are incompletely understood. Several reports have shown that phorbol esters protect neurons from excitotoxicity, the process by which excessive receptor activation produces cell death [32,33]. By contrast, others have noted that phorbol esters induce oxidative stress and cell death [34]. PKC also activates several pathways involved in protein synthesis and synaptogenesis [35], including those mediated by embryonic lethal, abnormal vision (ELAV) mRNA-stabilizing proteins, nuclear factor (NF)- κ B, α -secretases and neurotrophins.

Neurodegeneration

Some research indicates that PKC activation can reduce the levels of A β , the toxic peptide believed to be important in AD [36]. PKC activation via phorbol esters [37], bryostatin [36] or benzolactam derivatives [38] produces a dramatic increase in the activity of α -secretase, the enzyme that cleaves amyloid precursor protein (APP) to produce the nontoxic soluble sAPP α . This indicates that α -secretase might inhibit A β production, perhaps by competing for APP availability. Other reports indicate that PKC increases the activity of A β -degrading enzymes such as endothelin-converting enzyme [39,40], insulin-degrading enzyme or neprilysin. Another theory suggests that PKC regulates the formation of secretory vesicles containing sAPP β [41]. Phorbol esters also attenuate the neuronal apoptosis induced by exposure to A β peptide [42]. Notably, this protection can be blocked by PKC inhibitors [42]. PKC activators, administered over a period of several months, provided statistically significant benefits to transgenic mice overexpressing a mutant form of APP (V717I) [36]. These mice develop amyloid plaques and 50% die before reaching 6 months of age. Bryostatin administration pro-

longs their life expectancy, increases sAPP α levels and decreases A β levels in the brain. Although PKC levels were not measured in this experiment, large increases in α -secretase activity were observed [36]. Bryostatin also has cognitive-enhancing effects in normal rats [2].

Paradoxically, phorbol esters can increase the synthesis of APP, the parent molecule of A β [43]. However, although therapeutic doses of PKC activators decrease A β levels, at least in animals, chronic treatment at higher doses could actually increase A β generation either by downregulating PKC or increasing APP synthesis. Indeed, a recent report indicates that 1 μ M phorbol 12,13-dibutyrate applied for long periods can increase A β levels in COS-7 cells, presumably by downregulating PKC [44].

One complication in the therapeutic use of PKC activators is the part that PKC plays in apoptosis. Although PKC signaling is usually anti-apoptotic, PKC δ and PKC θ are often regarded as having a pro-apoptotic function because they are components of the caspase apoptosis pathway [45]. PKC δ is proteolyzed by caspase 3, creating an unregulated, active form of PKC δ that participates in processes leading to apoptotic cell death. PKC ϵ , by contrast, has an opposite role: its activation promotes proliferation and cell survival, and inhibits apoptosis. Therefore, if PKC activators are to achieve their full potential as weapons against neurodegeneration, both activation and downregulation of pro-apoptotic PKC isoforms must be minimized.

Neuroprotection

In rats, PKC activators reverse the neurological damage caused by a combination of ischemia and hypoxia, a treatment designed to mimic stroke [24]. When bryostatin was given to impaired rats 24 hours after the ischemic/hypoxic event, the learning rates, the numbers of neurons and the synaptic morphologies of the animals returned to normal. These reversal effects could be mediated by inhibition of apoptosis or release of neurotrophins. Bryostatin and phorbol esters can also activate neurite outgrowth of axotomized spiral ganglion neurons. This effect is blocked by inhibitors of PI3K and MEK (MAP kinase kinase) [46].

PKC could also be important in regulating neurotrophin production. PKC can activate α -secretase, which cleaves the extracellular domain of the p75 neurotrophin receptor leading to the formation of a 25 kDa intracellular domain. The biochemical effects of this cleavage remain unclear. On the one hand, the three inhibitors of axonal regeneration in myelinated neurons (Nogo, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein) activate PKC-dependent p75 proteolytic cleavage, resulting in inhibition of neurite outgrowth [47]. On the other hand, proteolytic cleavage of the p75 extracellular domain enables FGF-2 (fibroblast growth factor 2) to stimulate neurite outgrowth and branching [48]. Some reports indicate that PKC activation can lead to neuronal growth and synaptogenesis, perhaps by activating the p75 neurotrophin receptor or by increasing the production of neurotrophins such as nerve growth factor or brain-derived neurotrophic factor [46].

Memory

It has long been known that blocking protein synthesis prevents the formation of long-term memory [49]. PKC is

Review

also essential for learning [49]. However, at least in invertebrates, pretreatment with PKC activators eliminates the dependence of learning on protein synthesis. In *Hermisenda crassicornis*, a sea snail often used for studies of the neurobiology of memory, treatment with 0.27 nM bryostatin for two days before training prevents the protein synthesis inhibitor anisomycin from blocking long-term memory [35]. In rats trained in the water maze spatial memory task, bryostatin also increases the rate of learning and the number of mushroom spines in the hippocampus. Mushroom spines are the largest and most stable type of dendritic spines (small protrusions on dendrites that contain postsynaptic densities) and are believed to be important in memory. The effects of bryostatin are blocked by PKC inhibitors [1]; thus, PKC activation directly affects memory and the formation of mature synapses.

Tumor promotion

Perhaps the most dire potential consequence of PKC dysregulation is tumorigenesis. However, the tumor-promoting ability of PKC activators does not correlate with their ability to activate PKC. For example, bryostatin is 10–100 times more potent than phorbol esters at activating PKC. Nonetheless, in over a dozen clinical studies representing >1000 patients, in which investigators specifically looked for metastasis, remission, tumor size and tumor number, there are no reports of bryostatin increasing tumor growth. Thus, the absence of bryostatin tumor-promoting activity can be considered to have been empirically demonstrated. By contrast, phorbol esters are the most potent tumor promoters known. What could account for these differences?

One noticeable difference is the presence or absence of a heteroatom corresponding to the 3-ketone in PMA (Figure 2b; Table 1). The tumor-promoting phorbol esters ingenol, teleocidins and aplysiatoxin all possess an additional oxygen at the 3 position, whereas the natural agonist DAG and the antitumor agent bryostatin do not [12,50]; in gnidimacrin, another antitumor agent, this position is esterified. However, although a 3-keto group might be essential for tumor-promoting activity, it is not sufficient. Both the 4 α -isomer of PMA, which is completely inactive, and 12-deoxyphorbol 13-phenylacetate, a weak activator of PKC β I and PKC η [51], possess this group. Indeed, several other functional groups (e.g. on the C4, C9 and C20 positions of phorbol), in addition to the lipid tail, must all have the proper 3D configuration to activate PKC and produce biological effects.

Although most researchers regard PKC activation as the primary mode of action of phorbol esters, much evidence indicates that isozyme-specific PKC downregulation, particularly of PKC δ , contributes more than activation to their tumor-promoting activity [52,53]. As mentioned earlier, whereas bryostatin and phorbol esters are approximately equal in their ability to activate different PKC isozymes, phorbol esters are more effective at downregulating PKC δ . Bryostatin prevents phorbol-ester-induced downregulation of PKC δ , but not of PKC α or PKC ϵ [52]. In glioma cell lines, PKC δ overexpression inhibits cell proliferation [54], whereas PKC α overexpression promotes proliferation. PKC δ also induces apoptosis by activating

expression of tumor-suppressor proteins [55]. This finding is consistent with a role for PKC δ as a tumor inhibitor [56,57]. By contrast, PKC β II overexpression predisposes animals to colon cancer. This effect is blocked by a MEK inhibitor, indicating a role of the Ras–MAP kinase pathway in this process [56]. Specific PKC β inhibitors, such as enzastaurin, are now undergoing clinical trials in patients with a variety of cancer types [56].

There is little evidence to indicate any correlation, positive or negative, between the tumor-promoting and neuroprotective effects of PKC activators. However, one factor that might be important is isozyme specificity. Bryostatin most strongly activates PKC α , PKC δ and PKC ϵ and promotes PKC β translocation to the nucleus. PKC ϵ activation, in particular, seems to have several neuroprotective effects owing to its anti-apoptotic function. Indeed, PKC ϵ activation or overexpression prevents apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family of cytokines [58]. PKC activation also inhibits TNF receptor-associated death domain protein (TRADD) recruitment to TNF receptor 1, and inhibits TNF-induced I κ B- α (inhibitor of NF- κ B) phosphorylation. Phorbol esters also block TNF-mediated reactive oxygen species production [59]. This probably involves PKC δ because this isoform associates with the TNF receptor 1 in response to TNF and is required to inhibit TNF-mediated apoptosis [60]. Thus, PKC activation disrupts the formation of the TNF receptor 1 signaling complex and inhibits both apoptotic and necrotic cell death. By contrast, PKC δ activation often induces apoptosis (for review, see Ref. [61]). These differing results indicate that PKC δ -specific activators would be useful as tools in studying cancer, whereas PKC ϵ -specific activators such as DCP-LA might be useful in treating neurological diseases such as AD or stroke.

Antitumor activity

Early researchers discovered that phorbol esters are effective tumor promoters. This led to the conclusion that PKC activation promotes tumor-cell growth. Subsequently, it was reasoned that using PKC activators to downregulate PKC might be useful in treating cancer (for review, see Ref. [56]). Unfortunately, the predicted antitumor effects of broad-spectrum PKC activators such as bryostatin have so far failed to materialize in clinical trials. Indeed, downregulating PKC is a risky strategy: it could deregulate the cell cycle, with dire consequences [62].

Cell cycling

PKC is a key regulator of cell cycling [63] (Figure 3). The role of PKC in the cell cycle is somewhat complicated because different isozymes perform different, and sometimes opposite, functions. However, it is clear that in most cells PKC activity inhibits the cell cycle [63–65]. PKC interacts with cyclins, the principal regulators of the cell cycle, at three points. Normally, phosphorylation inactivates the tumor-suppressor retinoblastoma protein (Rb), causing it to dissociate from the complex between Rb, the DNA-binding protein DP-1, and the eukaryotic transcription factor E2F, releasing the E2F transcription factor and driving the cell cycle into S phase [66]. In early G1, PKC

Review

apoptosis. If care is taken to avoid downregulation, PKC activators could become a powerful weapon in the battle against neurodegenerative disease.

References

- Hongpaisan, J. and Alkon, D.L. (2007) A structural basis for enhancement of long-term associative memory in single dendritic spines regulated by PKC. *Proc. Natl. Acad. Sci. U. S. A.* 104, 19571–19576
- Sun, M.K. and Alkon, D.L. (2005) Dual effects of bryostatin-1 on spatial memory and depression. *Eur. J. Pharmacol.* 512, 43–51
- Podar, K. *et al.* (2007) The therapeutic role of targeting protein kinase C in solid and hematologic malignancies. *Expert Opin. Investig. Drugs* 16, 1693–1697
- Lee, H.W. *et al.* (1997) Bryostatin 1 and phorbol ester down-modulate protein kinase C- α and - ϵ via the ubiquitin/proteasome pathway in human fibroblasts. *Mol. Pharmacol.* 51, 439–447
- Seki, T. *et al.* (2005) Phosphorylation of PKC activation loop plays an important role in receptor-mediated translocation of PKC. *Genes Cells* 10, 225–239
- Newton, A.C. (2001) Protein kinase C: Structural and spatial regulation by phosphorylation, cofactor, and macromolecular interactions. *Chem. Rev.* 101, 2353–2364
- Schechtman, D. and Mochly-Rosen, D. (2001) Adaptor proteins in protein kinase C-mediated signal transduction. *Oncogene* 20, 6339–6347
- Kronfeld, I. *et al.* (2000) Phosphorylation of protein kinase C δ on distinct tyrosine residues regulates specific cellular functions. *J. Biol. Chem.* 275, 35491–35498
- Leontieva, O.V. and Black, J.D. (2004) Identification of two distinct pathways of protein kinase C α down-regulation in intestinal epithelial cells. *J. Biol. Chem.* 279, 5788–5801
- Prevostel, C. *et al.* (2000) Protein kinase C α actively downregulates through caveolae-dependent traffic to an endosomal compartment. *J. Cell Sci.* 113, 2575–2584
- Zhang, G. *et al.* (1995) Crystal structure of the cys2 activator-binding domain of protein kinase C δ in complex with phorbol ester. *Cell* 81, 917–924
- Jeffrey, A.M. and Liskamp, R.M. (1986) Computer-assisted molecular modeling of tumor promoters: rationale for the activity of phorbol esters, teleocidin B, and aplisyatoxin. *Proc. Natl. Acad. Sci. U. S. A.* 83, 241–245
- Wender, P.A. *et al.* (1988) Modeling of the bryostatins to the phorbol ester pharmacophore on protein kinase C. *Proc. Natl. Acad. Sci. U. S. A.* 85, 7197–7201
- Lewin, N.E. *et al.* (1992) Binding of [³H]bryostatin 4 to protein kinase C. *Biochem. Pharmacol.* 43, 2007–2014
- Lorenzo, P.S. *et al.* (1999) Differential roles of the tandem C1 domains of protein kinase C δ in the biphasic down-regulation induced by bryostatin 1. *Cancer Res.* 59, 6137–6144
- Colón-González, F. and Kazanietz, M.G. (2006) C1 domains exposed: from diacylglycerol binding to protein-protein interactions. *Biochim. Biophys. Acta* 1761, 827–837
- Irie, K. *et al.* (2002) Establishment of a binding assay for protein kinase C isozymes using synthetic C1 peptides and development of new medicinal leads with protein kinase C isozyme and C1 domain selectivity. *Pharmacol. Ther.* 93, 271–281
- Giorgione, J. *et al.* (2003) Contribution of the C1A and C1B domains to the membrane interaction of protein kinase C. *Biochemistry* 42, 11194–11202
- Medkova, M. and Cho, W. (1999) Interplay of C1 and C2 domains of protein kinase C- α in its membrane binding and activation. *J. Biol. Chem.* 274, 19852–19861
- Ananthanarayanan, B. *et al.* (2003) Activation mechanisms of conventional protein kinase C isoforms are determined by the ligand affinity and conformational flexibility of their C1 domains. *J. Biol. Chem.* 278, 46886–46894
- Stahelin, R.V. *et al.* (2005) Diacylglycerol-induced membrane targeting and activation of protein kinase C: mechanistic differences between protein kinases C δ and C ϵ . *J. Biol. Chem.* 280, 19784–19793
- Kuzirian, A.M. *et al.* (2006) Bryostatin enhancement of memory in *Hermisenda*. *Biol. Bull.* 210, 201–214
- Marshall, J.L. *et al.* (2002) Phase I study of prolonged infusion bryostatin-1 in patients with advanced malignancies. *Cancer Biol. Ther.* 1, 409–416
- Sun, M.K. *et al.* (2008) Post-stroke neuronal rescue and synaptogenesis mediated *in vivo* by PKC in adult brains. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13620–13625
- Verdager, N. *et al.* (1999) Ca²⁺ bridges the C2 membrane-binding domain of protein kinase C α directly to phosphatidyserine. *EMBO J.* 18, 6329–6388
- Cavallaro, S. *et al.* (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. *Proc. Natl. Acad. Sci. U. S. A.* 94, 9669–9673
- Zhao, W. *et al.* (1999) Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. *J. Biol. Chem.* 274, 34893–34902
- Reuss, B. *et al.* (2003) Fibroblast growth factors and their receptors in the central nervous system. *Cell Tissue Res.* 313, 139–157
- Meinhardt, G. *et al.* (2002) Effect of novel modulators of protein kinase C activity upon chemotherapy-induced differentiation and apoptosis in myeloid leukemic cells. *Anticancer Drugs* 13, 725–733
- Terry, R.D. (2000) Cell death or synaptic loss in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 59, 1118–1119
- Scheff, S.W. *et al.* (2006) Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. *Neurobiol. Aging* 27, 1372–1384
- Tomimatsu, N. and Arakawa, Y. (2008) Protein kinase C-mediated protection of motoneurons from excitotoxicity. *Neurosci. Lett.* 439, 143–146
- Jung, Y.S. *et al.* (2005) Activation of protein kinase C- δ attenuates kainate-induced cell death of cortical neurons. *Neuroreport* 16, 741–744
- Noh, K.M. *et al.* (2000) A novel neuroprotective mechanism of riluzole: direct inhibition of protein kinase C. *Neurobiol. Dis.* 7, 375–383
- Alkon, D.L. *et al.* (2007) PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer's disease. *Trends Pharmacol. Sci.* 28, 51–60
- Etcheberrigaray, R. *et al.* (2004) Therapeutic effect of PKC activators on Alzheimer's disease transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 101, 11141–11146
- Buxbaum, J.D. *et al.* (1998) Evidence that tumor necrosis factor α converting enzyme is involved in regulated α -secretase cleavage of the Alzheimer amyloid protein precursor. *J. Biol. Chem.* 273, 27765–27767
- Yang, H.Q. *et al.* (2007) New protein kinase C activator regulates amyloid precursor protein processing *in vitro* by increasing α -secretase activity. *Eur. J. Neurosci.* 26, 381–391
- Choi, D.S. *et al.* (2006) PKC ϵ increases endothelin converting enzyme activity and reduces amyloid plaque pathology in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8215–8220
- Greenberg, D.A. (2006) Another weapon against amyloid. *Proc. Natl. Acad. Sci. U. S. A.* 103, 7943–7944
- Xu, H. *et al.* (1995) Regulated formation of Golgi secretory vesicles containing Alzheimer β -amyloid precursor protein. *J. Biol. Chem.* 270, 23243–23245
- Behrens, M.M. *et al.* (1999) Prevention of neuronal apoptosis by phorbol ester-induced activation of protein kinase C: blockade of p38 mitogen-activated protein kinase. *Neuroscience* 94, 917–927
- Trejo, J. *et al.* (1994) A direct role for protein kinase C and the transcription factor Jun/AP-1 in the regulation of the Alzheimer's β -amyloid precursor protein gene. *J. Biol. Chem.* 269, 21682–21690
- da Cruz, E. *et al.* (2009) Enhanced generation of Alzheimer's amyloid- β following chronic exposure to phorbol ester correlates with differential effects on α and ϵ isozymes of protein kinase C. *J. Neurochem.* 108, 319–330
- Gutcher, I. *et al.* (2003) The isoform-specific regulation of apoptosis by protein kinase C. *Cell. Mol. Life Sci.* 60, 1061–1070
- Lallemend, F. *et al.* (2005) Activation of protein kinase C β 1 constitutes a new neurotrophic pathway for deafferented spiral ganglion neurons. *J. Cell Sci.* 118, 4511–4525
- Domeniconi, M. *et al.* (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* 46, 849–855
- Ahmed, Z. *et al.* (2006) TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhibits outgrowth and promotes

- branching of neurites in the presence of inhibitory CNS myelin. *FASEB J.* 20, 1939–1941
- 49 Bonini, J.S. *et al.* (2007) On the participation of hippocampal PKC in acquisition, consolidation and reconsolidation of spatial memory. *Neuroscience* 147, 37–45
- 50 Kozikowski, A.P. *et al.* (1997) Modeling, chemistry, and biology of the benzolactam analogues of indolactam V (ILV). 2. Identification of the binding site of the benzolactams in the CRD2 activator-binding domain of PKC δ and discovery of an ILV analogue of improved isozyme selectivity. *J. Med. Chem.* 40, 1316–1326
- 51 Saraiva, L. *et al.* (2004) Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay. *Eur. J. Pharmacol.* 491, 101–110
- 52 Lu, Z. *et al.* (1997) Tumor promotion by depleting cells of protein kinase C δ . *Mol. Cell. Biol.* 17, 3418–3428
- 53 Scaglione-Sewell, B. *et al.* (1998) Decreased PKC- α expression increases cellular proliferation, decreases differentiation, and enhances the transformed phenotype of CaCo-2 cells. *Cancer Res.* 58, 1074–1081
- 54 Mandil, R. *et al.* (2001) Protein kinase C α and protein kinase C δ play opposite roles in the proliferation and apoptosis of glioma cells. *Cancer Res.* 61, 4612–4619
- 55 Ryer, E.J. *et al.* (2005) Protein kinase C δ induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. *J. Biol. Chem.* 280, 35310–35317
- 56 Griner, E.M. and Kazanietz, M.G. (2007) Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* 7, 281–294
- 57 Jackson, D.N. and Foster, D.A. (2004) The enigmatic protein kinase C δ : complex roles in cell proliferation and survival. *FASEB J.* 18, 627–636
- 58 Harper, N. *et al.* (2003) Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. *J. Biol. Chem.* 278, 44338–44347
- 59 Byun, H.S. *et al.* (2006) Phorbol 12-myristate 13-acetate protects against tumor necrosis factor (TNF)-induced necrotic cell death by modulating the recruitment of TNF receptor 1-associated death domain and receptor-interacting protein into the TNF receptor 1 signaling complex: Implication for the regulatory role of protein kinase C. *Mol. Pharmacol.* 70, 1099–1108
- 60 Kilpatrick, L.E. *et al.* (2004) Selective regulation by δ -PKC and PI3-kinase in the assembly of the antiapoptotic TNFR-1 signaling complex in neutrophils. *Am. J. Physiol. Cell Physiol.* 287, C633–C642
- 61 Reyland, M.E. (2007) Protein kinase C δ and apoptosis. *Biochem. Soc. Trans.* 35, 1001–1004
- 62 Malumbres, M. and Carnero, A. (2003) Cell cycle deregulation: a common motif in cancer. *Prog. Cell Cycle Res.* 5, 5–18
- 63 Black, J.D. (2000) Protein kinase C-mediated regulation of the cell cycle. *Front. Biosci.* 5, D406–D423
- 64 Nakashima, S. (2002) Protein kinase C α (PKC α): regulation and biological function. *J. Biochem.* 132, 669–675
- 65 Livneh, E. and Fishman, D. (1997) Linking protein kinase C to cell-cycle control. *Eur. J. Biochem.* 248, 1–9
- 66 Gavrieliades, M.V. *et al.* (2004) Protein kinase C and prostate carcinogenesis: targeting the cell cycle and apoptotic mechanisms. *Curr. Drug Targets* 5, 431–443
- 67 Kedei, N. *et al.* (2004) Characterization of the interaction of ingenol 3-angelate with protein kinase C. *Cancer Res.* 64, 3243–3255
- 68 Vogt, G. *et al.* (1999) Tumor promoting diterpenes from *Euphorbia leuconeura* L. *Phytochemistry* 51, 289–295
- 69 Fujiki, H. and Sugimura, T. (1987) New classes of tumor promoters: teleocidin, aplysiatoxin, and palytoxin. *Adv. Cancer Res.* 49, 223–264
- 70 Irie, K. *et al.* (2004) Indolactam and benzolactam compounds as new medicinal leads with binding selectivity for C1 domains of protein kinase C isozymes. *Curr. Pharm. Des.* 10, 1371–1385
- 71 Yoshida, M. *et al.* (1998) Mechanism of antitumor action of PKC activator, gnidimacrin. *Int. J. Cancer* 77, 243–250
- 72 Shao, L. *et al.* (2001) Iridals are a novel class of ligands for phorbol ester receptors with modest selectivity for the RasGRP receptor subfamily. *J. Med. Chem.* 44, 3872–3880
- 73 Gilbert, B.A. *et al.* (1995) Farnesyl thiotriazole, a potent neutrophil agonist and structurally novel activator of protein kinase C. *Biochemistry* 34, 3916–3920
- 74 Lee, J. *et al.* (2006) Branched diacylglycerol-lactones as potent protein kinase C ligands and α -secretase activators. *J. Med. Chem.* 49, 2028–2036
- 75 Kerr, D.E. *et al.* (1987) Structural requirements of diacylglycerols for binding and activating phospholipid-dependent, Ca⁺⁺-sensitive protein kinase. *Biochem. Biophys. Res. Commun.* 148, 776–782
- 76 Kanno, T. *et al.* (2006) The linoleic acid derivative DCP-LA selectively activates PKC- ϵ , possibly binding to the phosphatidylserine binding site. *J. Lipid Res.* 47, 1146–1150
- 77 Shearman, M.S. *et al.* (1989) Selective activation of the γ -subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoygenase metabolites. *FEBS Lett.* 243, 177–182
- 78 Ito, M. *et al.* (1986) N-(6-phenylhexyl)-5-chloro-1-naphthalene-sulfonamide, a novel activator of protein kinase C. *Biochemistry* 25, 4179–4184
- 79 Takekoshi, S. *et al.* (1995) Activation of protein kinase C by oxidized diacylglycerols. *Biochem. Biophys. Res. Commun.* 217, 654–660
- 80 Kawahara, Y. *et al.* (1986) Activation of protein kinase C by the action of 9,11-epithio-11,12-methano-thromboxane A2 (STA2), a stable analogue of thromboxane A2, in human platelets. *Thromb. Res.* 41, 811–818
- 81 Mitra, J. and Enders, G.H. (2004) Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. *Oncogene* 23, 3361–3367