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Tuning the signalling output of protein kinase C

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Abstract

Precise control of the amplitude of protein kinase C (PKC) signalling is essential for cellular homoeostasis, and disruption of this control leads to pathophysiological states such as cancer, neurodegeneration and diabetes. For conventional and novel PKC, this amplitude is meticulously tuned by multiple inputs that regulate the amount of enzyme in the cell, its ability to sense its allosteric activator diacylglycerol, and protein scaffolds that co-ordinate access to substrates. Key to regulation of the signalling output of most PKC isoenzymes is the ability of cytosolic enzyme to respond to the membrane-embedded lipid second messenger, diacylglycerol, in a dynamic range that prevents signalling in the absence of agonists but allows efficient activation in response to small changes in diacylglycerol levels. The present review discusses the regulatory inputs that control the spatiotemporal dynamics of PKC signalling, with a focus on conventional and novel PKC isoenzymes.

Introduction

The protein kinase C (PKC) family transduces a multitude of signals that control diverse cellular processes such as proliferation, migration, invasion, differentiation, apoptosis, transcription and translation. Therefore aberrant PKC activity or localization has been linked to numerous diseases, most notably cancer, neurodegeneration and diabetes [1]. This serine/threonine kinase family belongs on the AGC kinase branch of the kinome [2] and comprises nine genes that share a similar architecture with an N-terminal regulatory moiety and a C-terminal kinase domain (Figure 1). PKCs are classified according to the second messengers that regulate their activity. Conventional PKCs (cPKCs: α, β and γ) contain tandem C1 domains that bind diacylglycerol (DAG) and phosphatidylserine (PS) and a C2 domain that binds anionic phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP_2), in a Ca^{2+}-dependent manner. Novel PKCs (nPKCs: δ, ε, η, θ) contain tandem C1 domains that bind DAG and a novel C2 domain that is Ca^{2+}-unresponsive and does not assist in membrane binding. Atypical PKCs (aPKCs: ι and ζ) have an atypical C1 domain that does not bind DAG and lack a C2 domain altogether, but instead contain a PB1 domain that mediates protein–protein interactions. aPKCs and PKCα also contain a C-terminal PDZ ligand that mediates protein–protein interactions and thus affects scaffolding and localization of these isoenzymes.

Regulation by priming phosphorylation

Phosphorylation is absolutely critical (i) to render PKC in a catalytically competent conformation and (ii) to protect PKC from degradation [3]. In contrast with many other kinases, the phosphorylation of PKC is constitutive and thus its activity is not acutely regulated by phosphorylation. Rather, cellular levels of PKC are directly regulated by its phosphorylation. cPKCs and nPKCs are constitutively phosphorylated at three conserved residues: the activation loop, the turn motif and the hydrophobic motif [4] (Figure 1). aPKCs are also phosphorylated at the activation loop and turn motif, but contain a phosphomimetic glutamic acid at the hydrophobic motif. The first priming phosphorylation on

Key words: conformational change, priming phosphorylation, protein kinase C (PKC), scaffold, second messenger.

Abbreviations: AKAP, A-kinase-anchoring protein; aPKC, atypical PKC; cPKC, conventional PKC; DAG, diacylglycerol; nPKC, novel PKC; PHLPP, PH domain leucine-rich repeat protein phosphatase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RACK, receptor for activated C-kinase; RINCK, RING-finger protein that interacts with C kinase.

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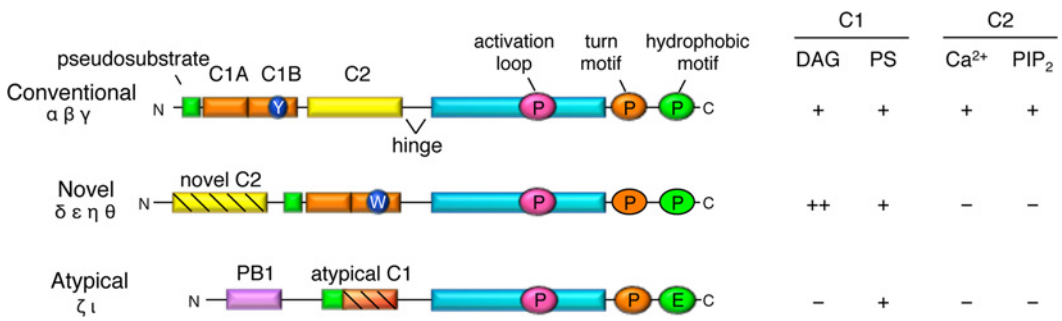
Figure 1 | Domain composition of the three classes of PKC

Conventional PKCs (α, β and γ) contain an N-terminal pseudosubstrate (green), tandem C1 domains (orange) that bind diacylglycerol (DAG) and phosphatidylserine (PS), a C2 domain (yellow) that binds anionic phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP_2), in a Ca^{2+}-dependent manner and a C-terminal kinase domain (cyan). Novel PKCs (δ, ε, η and θ) have a similar domain composition except that their C2 domain cannot bind Ca^{2+} or PIP_2 and their C1B domain has ~100-fold higher affinity for DAG due to having a tryptophan residue at position 22 in the domain, as opposed to a tyrosine residue as the C1B domain of cPKCs has [15]. Atypical PKCs (ζ and ι) have a protein-binding module, the PB1 domain, and an atypical C1 domain that cannot bind DAG. All PKCs are phosphorylated at three conserved sites: the activation loop within the kinase domain and the turn and hydrophobic motifs within the C-terminal tail (except for atypical PKCs which have a phosphomimetic Glu at the hydrophobic motif). The table on the right summarizes the second messengers that bind to each of the classes of PKC, with + representing binding, ++ representing binding with ~100-fold higher affinity, and − representing lack of binding.



	C1		C2	
	DAG	PS	Ca^{2+}	PIP_2
Conventional	+	+	+	+
Novel	++	+	−	−
Atypical	−	+	−	−

PKC occurs at the activation loop within the kinase domain and is catalysed by the phosphoinositide-dependent kinase, PDK-1 [5,6]. Phosphorylation at this site properly aligns residues within the active site for catalysis, an event that induces two tightly-coupled and ordered phosphorylations on the C-terminal tail: phosphorylation at the turn motif and intramolecular autophosphorylation at the hydrophobic motif. For cPKCs and nPKCs such as PKCε, but not PKCδ, these phosphorylation events require mTORC2; however, whether mTOR is the direct kinase for this site in cells remains controversial [7–10]. Phosphorylation at the hydrophobic motif controls the stability of the enzyme. Indeed, dephosphorylation of this site is the first step in the degradation of PKC as it destabilizes PKC and promotes its degradation. The PH domain leucine-rich repeat protein phosphatase (PHLPP) directly dephosphorylates the hydrophobic motif of PKCs, an event that requires the PH domain of PHLPP [11]. Thus loss of PHLPP in the cell leads to an increase in steady state PKC levels. Conversely, PKC levels are low in cells in which any of the phosphorylation steps have been perturbed, such as by loss of mTORC2 or PDK-1 [8,12]. Thus priming phosphorylations regulate the steady-state levels of PKC but not its acute agonist-dependent activity; instead, the spatial and temporal dynamics of PKC signalling are regulated by second messengers.

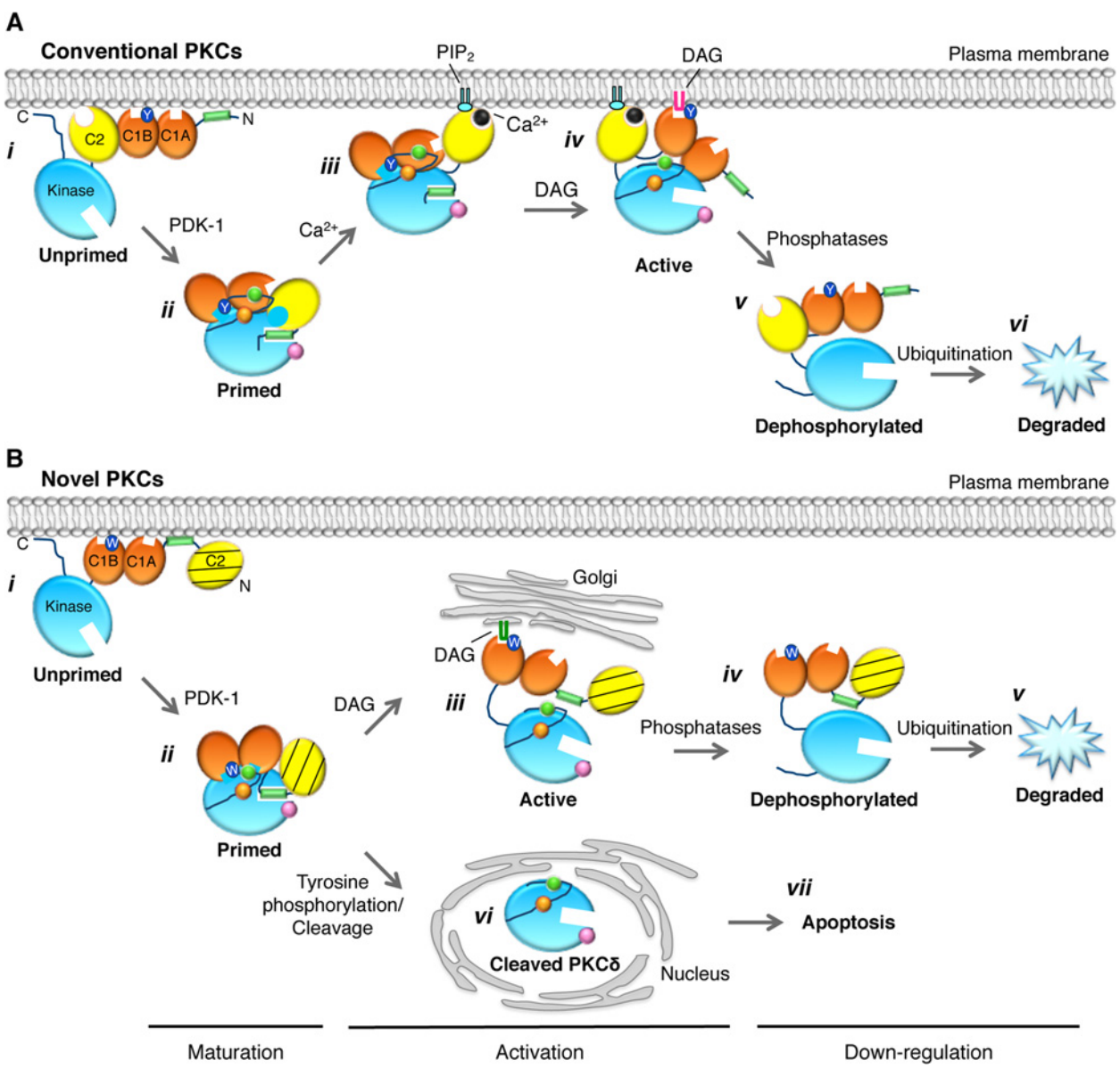
Regulation by second messengers

Although phosphorylated PKC is catalytically competent, an autoinhibitory pseudosubstrate binds the substrate-binding cavity to maintain PKC in an inactive conformation until the appropriate second messengers bind. cPKCs are allosterically activated by binding to two second messengers: Ca^{2+} and DAG (Figure 2A). Binding of Ca^{2+} to the C2 domain targets the kinase to the plasma membrane through (i) hydrophobic interactions that drive binding to the membrane, and (ii) electrostatic interactions with anionic phospholipids, including PIP_2, that contribute to retention of the C2 domain onto membranes [13]. Once at the membrane, one of the C1 domains of PKC is positioned to find and bind DAG, an event that provides the necessary energy to expel the pseudosubstrate and activate PKC [14]. nPKCs are activated solely by DAG (Figure 2B), whereas aPKCs do not respond to either of these second messengers and their activity is instead regulated by protein–protein interactions.

Differential binding of second messengers to cPKCs versus nPKCs leads to substantial differences in their spatiotemporal dynamics of signalling. First, cPKCs and nPKCs predominantly translocate to different membranes (Figure 2). cPKCs translocate to, and are active at, the plasma membrane because their Ca^{2+}-bound C2 domain pre-targets them to the plasma membrane-localized lipid PIP_2, where they are retained following DAG binding [13] (Figure 2A). nPKCs do not have a Ca^{2+}-sensing C2 domain to pre-target them to the plasma membrane; instead, their C1B domain has an approximately 100-fold higher affinity for DAG due to the presence of a tryptophan residue at position 22 in the domain, compared with the C1B domain of cPKCs that contains a tyrosine residue at that position (Figure 1) [15]. Consequently, nPKCs translocate to DAG-rich endomembranes such as the Golgi (Figure 2B). Indeed, impairing the Ca^{2+}-binding ability of the C2 domain of

Figure 2 | Regulation of conventional and novel protein kinase C

(**A**) Model showing the life cycle of conventional PKC (cPKC). (***i***) Unprimed cPKC is in a membrane-associated, open conformation in which both its C1A and C1B domains are fully exposed. (***ii***) Upon priming phosphorylation at its activation loop (pink circle) by PDK-1, followed by phosphorylation at the turn motif (orange circle) and the hydrophobic motif (green circle), cPKC matures into a closed conformation in which both the C1A and C1B domains become masked, the pseudosubstrate binds the substrate-binding site, and the primed enzyme localizes to the cytosol. This masking of the C1 domains prevents pre-targeting of cPKC to membranes in the absence of agonist-evoked increases in DAG, thus preventing basal signalling. (***iii***) In response to agonists that promote PIP_2 hydrolysis, cPKC is recruited to the plasma membrane in a Ca^{2+}-dependent manner. (***iv***) This pre-targeting to the plasma membrane facilitates binding to DAG, predominantly via the C1B domain, which expels the pseudosubstrate from the substrate-binding cavity, thereby activating PKC. (***v***) Dephosphorylation of activated cPKC allows it to regain the open conformation of unprimed PKC. (***vi***) Ubiquitination of cPKCs leads to its proteasome-mediated degradation, thus terminating signalling. (**B**) Model showing life cycle of novel PKC (nPKC). (***i***) Unprimed nPKC is also in an open conformation that associates with membranes. (***ii***) Priming phosphorylations induce a closed conformation with both C1 domains masked. (***iii***) In response to agonists that produce DAG, nPKC is recruited to and activated at the DAG-rich Golgi via its higher DAG affinity C1B domain (due to the presence of a tryptophan residue at position 22 within the domain). (***iv***) Activated nPKC is dephosphorylated, (***v***) ubiquitinated and degraded. (***vi***) The novel PKCδ can also be activated by tyrosine phosphorylation and caspase cleavage. (***vii***) Nuclear-localized PKCδ can induce apoptosis.



PKCα forces it to the Golgi instead, as the C1 domain interaction becomes the dominant force [13]. Secondly, the kinetics of activation differ between cPKCs and nPKCs. cPKCs display rapid but transient activity at the plasma membrane that tracks with the initial Ca^{2+} release [16]. In fact, the kinetics of cPKC activation closely follow Ca^{2+} levels [17]. PKCβII exhibits oscillatory translocation to the plasma membrane in response to Ca^{2+} oscillations produced by histamine stimulation of HeLa cells. This, in turn, leads to oscillatory phosphorylation of membrane-localized substrates that tracks Ca^{2+} levels with a slight lag. nPKCs, on the other hand, are activated with slower kinetics because they do not respond to this fast Ca^{2+} release [16]. Thirdly, the duration of PKC activity, which is partially controlled by the persistence of the second messengers at a particular subcellular location, also differs among the PKCs. For example, DAG levels are more sustained at the Golgi than the plasma membrane, leading to prolonged nPKC activity at the Golgi compared with the short-lived cPKC activity at the plasma membrane [16]. Thus second messengers precisely dictate the kinetics, magnitude, duration, and location of cPKC and nPKC activity and are responsible for the apparent differences between them.

PKC levels, and thus PKC activity, are exquisitely controlled by various mechanisms both under basal conditions and after agonist stimulation. Under basal conditions, an E3 ligase for PKC, RING-finger protein that interacts with C kinase (RINCK), controls the amplitude of PKC signalling by regulating its levels [18]. RINCK interacts with the C1A domain of PKC and induces its ubiquitination and thus its degradation. PKCα activity was also shown to be regulated, under basal conditions, through an interaction with DAG kinase ζ, which prevents its activation by locally metabolizing DAG [19]. Only under stimulated conditions in which enough DAG is locally produced is PKCα activated, allowing it to phosphorylate DAG kinase ζ, thereby causing disassociation of the two proteins. However, agonist stimulation ultimately leads to termination of PKC signalling through various mechanisms. For example, PKC signalling is quickly terminated by the clearance of the respective second messengers, but also by agonist-induced down-regulation of the enzyme. Several mechanisms control this down-regulation. First, the peptidyl-prolyl isomerase Pin1 controls the isomerization of the turn motif (LTP), an event that is required to allow dephosphorylation of this site [20]. Thus Pin1 converts PKC into a down-regulation-capable species. Secondly, this species of active PKC can be dephosphorylated by PHLPP (hydrophobic motif) causing it to be shunted to the detergent-insoluble fraction where it is further dephosphorylated by okadaic acid-sensitive phosphatases such as protein phosphatase 2A (activation loop and turn motif), ubiquitinated and ultimately degraded by the proteasome [21]. Agonist-induced proteasome-mediated degradation of PKCα can, however, also occur via ubiquitination of plasma membrane-localized, fully primed PKC [22,23]. Additionally, phosphorylated PKCα can also be internalized through lipid raft-mediated endocytic pathways and degraded by the lysosome [23,24]. These mechanisms desensitize PKC signalling by regulating PKC levels, thus providing another means of exquisite control.

Regulation independent of second messengers

Particular cPKCs and nPKCs can also be activated independently of second messengers, adding to the complexity of PKC signalling. For example, certain PKCs can be activated by the accumulation of reactive oxygen species, which are often elevated in diseases such as cancer, cardiovascular disease, and neurodegeneration [25]. Specifically, H_2O_2 causes oxidation of cysteine residues within the C1B domain of PKCγ, inducing conformational changes that release PKCγ from its scaffold, leading to its translocation to the plasma membrane and subsequent DAG-independent activation [26,27]. PKCδ is phosphorylated at multiple tyrosine residues by Src family kinases in response to acute stimulation of cells by H_2O_2, epidermal growth factors, or platelet-derived growth factor. Tyrosine phosphorylation can induce the DAG-independent activation of PKCδ, in the absence of membrane translocation, and can alter its subcellular localization [28,29]. For example, tyrosine phosphorylation of PKCδ at Tyr^{64} and Tyr^{155} in response to apoptotic stimuli, such as H_2O_2 and etoposide, induces a conformational change that exposes its nuclear localization sequence and chaperone-binding site, allowing its import into the nucleus where it can induce apoptosis [30]. H_2O_2-induced tyrosine phosphorylation at Tyr^{311} has also been proposed to activate PKCδ by inducing caspase-3 cleavage between its regulatory and catalytic domains, resulting in a nuclear-localized, uninhibited catalytic domain [31]. Therefore these agonist-induced phosphorylations and conformational changes can activate PKCs independently of second messengers by either releasing PKCs from scaffolds or by exposing binding sites for scaffolds to bind.

Regulation by conformational changes

PKCs are under precise conformational control, particularly during maturation, adding another level of regulation. When first synthesized, PKC is in an open conformation that has both the C1A and C1B domains exposed (Figure 2). Upon phosphorylation at its three priming sites, PKC adopts a closed conformation that masks its C1 domains such that the lower affinity C1B domain is the predominant DAG binder. This conformational change optimizes PKC's dynamic range of signalling such that it is not activated by basal DAG but can readily detect and be activated by a small, local increase in DAG [32]. Phosphorylation of the C-terminal tail is critical in maintaining PKC in a closed inactive conformation, as lack of phosphorylation at these sites leads to PKC remaining in an open exposed conformation. Binding to its respective second messengers leads to another conformational change that results in expulsion of the autoinhibitory pseudosubstrate

and subsequent activation of PKC [35]. Similar to cPKCs, the pseudosubstrate and C1 domain of the aPKCζ also inhibit its activity through intramolecular interactions with the substrate-binding site and the αC helix, respectively [33,34].

Thus PKC undergoes conformational changes both during its maturation and during its activation in order to finely tune its activity.

Regulation by scaffolding

Scaffolding also plays an integral part in determining the precise location, duration and amplitude of PKC activity, as well as in establishing substrate specificity. Considering that there are multiple PKC isoenzymes expressed in the same cell that are activated by the same stimuli, scaffolds provide a level of functional selectivity. Among the PKC scaffolds are receptors for activated C-kinase (RACK) [36–38], 14-3-3 proteins [39,40] and A-kinase-anchoring proteins (AKAPs) [41,42].

Particular scaffolds augment PKC signalling, whereas others inhibit it. RACKs were the first scaffolds found to associate with active PKC. For PKCβII, this interaction occurs via its C2 domain and C-terminal tail and it stabilizes PKC's active conformation, thus enhancing its activity towards co-scaffolded substrates [36–38]. The phosphoserine/threonine binding protein 14-3-3 binds to a pair of phosphoserines within the hinge region of PKCε, leading to its activation [39]. However, scaffolds can also be inhibitory towards PKC by sequestering it away from its substrates or maintaining it in an inactive conformation. For example, in lens epithelial cells, 14-3-3ε binds PKCγ's C1B domain and controls both its activity and localization, thus regulating gap junction activity [40]. A subset of AKAPs, which were first identified to bind protein kinase A, has been shown to bind PKC. AKAP12 binds to and attenuates PKCα and PKCδ signalling, thereby preventing senescence and oncogenic transformation [41]. Similarly, AKAP5 inhibits PKC activity by binding to its catalytic pocket [42].

Scaffolds can also regulate the duration of PKC activity towards a substrate by co-scaffolding a phosphatase of the substrate. The phosphatase can thus rapidly dephosphorylate and attenuate signalling downstream of PKC substrates. Such an example is the co-ordination of PKC and protein phosphatase 2B/calcineurin on AKAP79/150 at the postsynaptic density in neurons [43]. Another AKAP (AKAP350/AKAP450) was proposed to act as a scaffold for the maturation of PKC [44]. This AKAP only associates with nascent PKCε at the Golgi/centrosome and this complex disassembles following PKCε maturation by phosphorylation. Interestingly, this complex also contains protein phosphatase 2A [45], which dephosphorylates PKC leading to its degradation. Perhaps PKC levels are dynamically controlled on this scaffold through regulation of its phosphorylation. Scaffolding of PKC also has clinical relevance because scaffolds have been shown to change the pharmacological profile of PKC. Specifically, ATP-competitive inhibitors were found to be ineffective against scaffolded PKC [42]. To explain how scaffolds amplify, accelerate and insulate PKC signalling, Greenwald et al. [46] proposed a stochastic state-switching model. In this model, the complex containing PKC, its substrate and its scaffold alters between inactive and active intermediate states, thus allowing phosphorylation of the substrate, even in the presence of active-site PKC inhibitors. Accordingly, scaffolds are able to precisely control PKC activity and to confer functional selectivity.

Concluding remarks

As PKC activity has to be precisely balanced at every subcellular location, its regulation is under intricate control. This control of the spatial and temporal dynamics of PKC signalling comes from regulation through various mechanisms, such as phosphorylation, binding to second messengers, conformational changes and binding to scaffolds. Perturbation of any of these mechanisms of control can lead to pathophysiological states. In fact, dysregulation of PKC activity has been observed in many diseases including cancer [47], diabetes [48] and neurodegenerative diseases such as Alzheimer's [49]. Changes in expression of PKC scaffolds, such as RACK1, as opposed to PKC itself, have also been found to dysregulate PKC signalling in Alzhemier's disease [50]. Therefore understanding all mechanisms through which PKC is regulated is key to developing novel therapeutics to restore PKC activity to physiological levels and to appropriate subcellular locations.

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