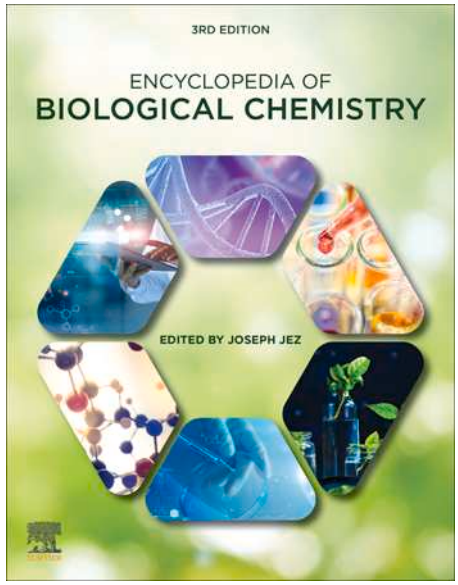

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Kinases/Phosphatases | Protein Kinase C Family☆

Alexandra C Newton, University of California, San Diego, CA, United States

Glossary

Diacylglycerol The membrane-retained lipid backbone released from phospholipids following activation of appropriate phospholipases, enzymes that hydrolyze phospholipids. Diacylglycerol is considered a second messenger because it transfers information from stimuli such as hormones to protein kinase C, which transduces the signal by phosphorylating protein substrates.

Kinase The class of enzymes that covalently transfer phosphate from adenosine triphosphate (ATP) to hydroxyl groups of proteins.

Phosphorylation The covalent attachment of phosphate from the cellular energy currency, ATP to hydroxyl residues of proteins, a modification that changes the properties of the protein.

Protein kinase C (PKC) is a family of enzymes that has a central role in transducing information from external stimuli to cellular responses (Nishizuka, 1986). Members of this family of serine/threonine kinases respond to signals that cause lipid hydrolysis. PKC isozymes phosphorylate an abundance of substrates, leading to both short-term cellular responses such as regulation of membrane transport and long-term responses such as memory and learning.

Historical Perspective

PKC was discovered in the late 1970s by Yasutomi Nishizuka and colleagues at Kobe University, Japan (Kikkawa, 2019). Their initial discovery was of a constitutively active enzyme that required only Mg^{2+} for activity (and hence was named protein kinase M, PKM). Further studies revealed that PKM was a proteolytic product of a full-length enzyme; this enzyme was named PKC because its enzymatic activity could be released by a Ca^{2+}-dependent protease. The subsequent discovery that PKC is activated by the phospholipid hydrolysis product, diacylglycerol, was a major finding in biology: it provided the molecular mechanism for how lipid hydrolysis, discovered 25 years earlier to be triggered by stimuli such as acetylcholine, couples to cellular signaling pathways.

But the discovery that catapulted research on PKC to the forefront of cellular signaling was the finding that it is the receptor for the potent tumor-promoting phorbol esters. Phorbol esters are present in the milky sap exuded from plants of the Euphorbiaceae family; the oil from the seeds of one member of this family, in particular Croton tiglium, has particularly strong irritant properties and, as such, has been used over the millennia for purposes as varied as poison for hunting arrows to medicinal purposes. In the 1960s, the active ingredient in the oil was found to be a family of diesters of the tetracyclic diterpene phorbol. Phorbol esters were shown to be extremely potent tumor promoters, and classic studies revealed that repetitive application of phorbol esters on the skin of mice allowed otherwise subthreshold amounts of carcinogens to promote tumors. The finding that PKC is the direct molecular target of phorbol esters placed this enzyme at the center of signaling pathways that control normal cell function and carcinogenesis.

PKC Family Members

There are 9 mammalian isozymes of PKC that share in common a carboxyl-terminal kinase domain linked to an amino-terminal regulatory moiety (**Fig. 1**) (Newton, 2018) (Cameron *et al.*, 2007). The regulatory moiety, in turn, contains a number of functional modules and it is the composition of these functional modules that further defines the three subfamilies of the PKC isozymes. These modules are an autoinhibitory-pseudosubstrate sequence that maintains the enzyme in an inactive conformation, and one or two membrane-targeting modules that direct PKC to the membrane following generation of the appropriate second messengers. Specifically, the C1 domain binds diacylglycerol and phorbol esters and the C2 domain binds Ca^{2+}; each event promotes the binding of the respective domain to membranes.

Conventional PKC isozymes (α, β, and γ), have tandem C1 domains and a C2 domain and respond to both diacylglycerol and Ca^{2+}. Novel PKC isozymes (δ, ε, η, and θ) have tandem C1 domains that bind diacylglycerol, but an impaired C2 domain that does not bind Ca^{2+}. These isozymes respond to increases in cellular diacylglycerol but not Ca^{2+}. Note that a change from a Tyr (found in conventional isozymes) to Trp in the C1b domain (the dominant diacylglycerol sensor) of novel isozymes confers an order of magnitude higher affinity for diacylglycerol, allowing novel isozymes to respond to signals that elevate only intracellular

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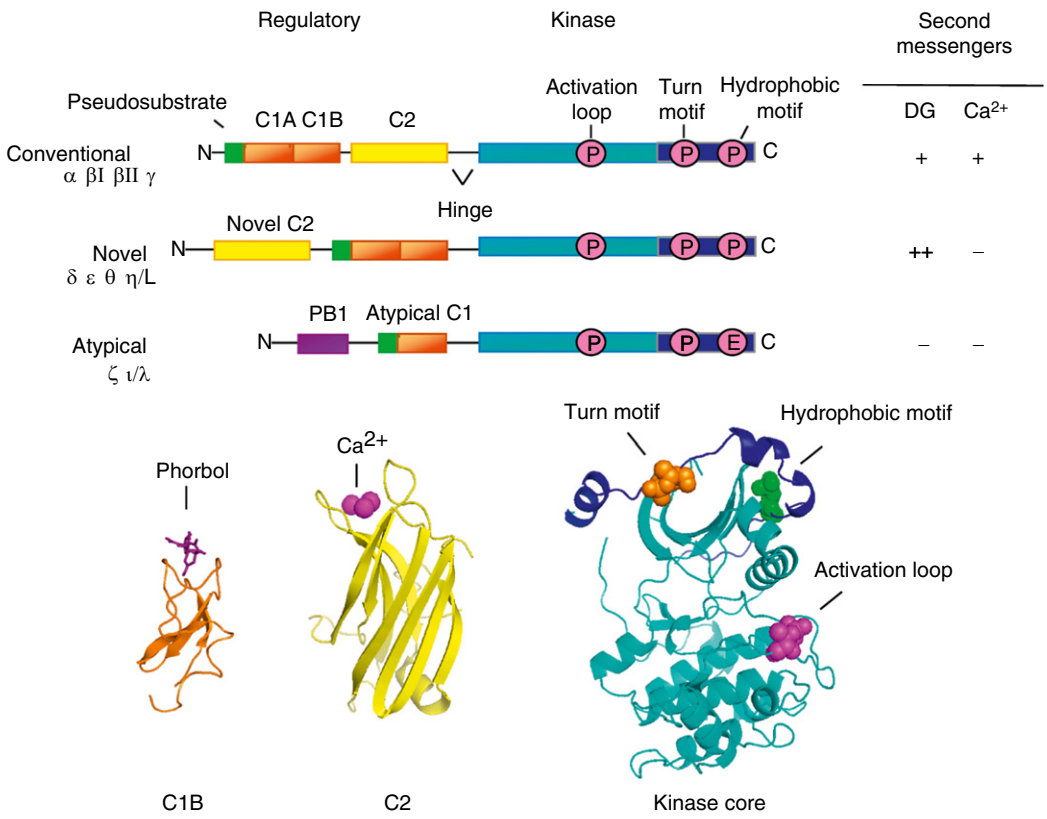

Fig. 1 Primary structure and domain composition of protein kinase C family members. The amino-terminal regulatory moiety contains the autoinhibitory-pseudosubstrate sequence (green), the C1 domain, which binds diacylglycerol/phorbol esters (orange; present as a tandem repeat in conventional and novel protein kinase C isozymes), and the C2 domain, which binds Ca^{2+} (yellow). The C2 domain in novel protein kinase Cs and the C1 domain in atypical protein kinase Cs are non-ligand-binding variants. Atypical isozymes contain a PB1 domain that mediates protein: protein interactions (purple). The carboxyl-terminal catalytic moiety contains the kinase core which has three phosphorylation sites, one on the activation loop segment (pink circle) and two on the carboxyl-terminal tail (blue): the turn motif (orange circle) and the hydrophobic motif (green circle; the negatively charged amino acid glutamate (E) occupies the phospho-acceptor position of the hydrophobic motif in atypical protein kinase Cs). The second messenger sensitivity is shown on the right of each isozyme subgroup; note that novel isozymes have a 10-fold increased affinity for diacylglycerol compared to conventional protein kinase C isozymes, allowing them to respond to signals that elevate only diacylglycerol and not both diacylglycerol and Ca^{2+}. The 3D structures of the domains are shown below the primary structure.

diacylglycerol (Dries *et al.*, 2007). Atypical PKC isozymes (ζ and ι/λ) have an impaired C1 domain and no C2 domain and bind neither diacylglycerol nor Ca^{2+}. Rather, they have a PB1 domain that mediates protein: protein interactions. Thus, stimuli that elevate intracellular diacylglycerol activate conventional and novel PKC family members, with conventional isozymes being additionally regulated by Ca^{2+}. Atypical isozymes are regulated by other mechanisms such as phosphorylation and protein interactions.

PKC Phosphorylation

Before PKC is competent to signal, it must first be processed by a series of ordered phosphorylations that are necessary for the enzyme to adopt a stable and autoinhibited conformation (Newton, 2018). These phosphorylations depend on two kinases: the phosphoinositide-dependent kinase, PDK-1, and the mammalian target of rapamycin (mTORC2) complex, which comprises the kinase mTOR, rictor, and a number of other interacting partners. These kinases have pivotal positions in cell signaling: PDK-1 catalyzes the activating phosphorylation to many other PKs, including the prosurvival kinase, Akt/PKB and mTORC2 plays an essential role in allowing Akt/PKB to become fully phosphorylated. Both kinases are necessary (but neither is sufficient) for the processing of PKC by phosphorylation, and lack of either one results in unphosphorylated PKC that is unstable and becomes degraded. Although the precise mechanism of phosphorylation is still being resolved, it appears that the first phosphorylation is catalyzed by PDK-1 on a conserved segment near the entrance to the active site referred to as the activation loop (**Fig. 1**), an event that structures the active site for substrate binding and catalysis. The phosphorylation of the activation loop by PDK-1 triggers two tightly coupled phosphorylations at two conserved positions in the carboxyl terminus, the turn motif and hydrophobic motif (**Fig. 1**). These latter phosphorylation events depend on mTORC2, but whether by direct phosphorylation or because mTORC2

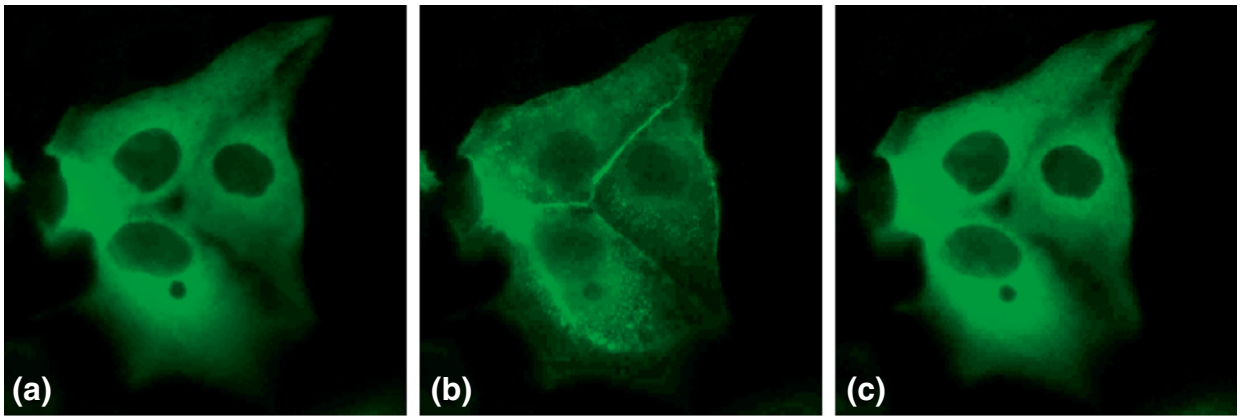

Fig. 2 Protein kinase C was visualized in cells by expression of a construct of protein kinase C fused to a naturally-fluorescent protein from the jellyfish *Aequorea victoria*, the green fluorescent protein (GFP). (a) Shows that protein kinase C is localized to the cytosol in unstimulated MDCK cells (diffuse fluorescence throughout the cell); (b) shows that protein kinase C translocates to the membrane (strong fluorescence intensity at cell peripheri) following 1 min of treatment with the agonist UTP, which induces phospholipid hydrolysis and generation of the two second messengers for protein kinase C: Ca^{2+} and diacyglycerol. (c) Shows that protein kinase C has redistributed back to the cytosol 5 min after UTP treatment; second messenger levels have returned to resting levels. Images courtesy of Jon Violin.

structures or positions PKC for C-terminal phosphorylations (which are mediated by intramolecular autophosphorylation in vitro) remains to be resolved. These phosphorylations lock PKC in its mature and catalytically competent conformation. This species is stable and has a half-life of days. It is this species of PKC that is transiently and reversibly activated by lipid hydrolysis and transduces signals.

PKC Translocation

Mature (i.e., phosphorylated) PKC is typically localized to the cytosol where it bounces on and off the membrane by diffusion-controlled mechanisms (although it is often scaffolded at specific locations, thus, increasing the local concentration for more effective signaling; see below). It is maintained in an inactive conformation because the pseudosubstrate sequence occupies the substrate-binding cavity. For conventional PKC isozymes, generation of Ca^{2+} and diacylglycerol target PKC to the membrane by binding the C2 and C1 domains and, thus, tethering the enzyme to membranes. The membrane-bound species adopts an active conformation by removal of the pseudosubstrate from the substrate-binding cavity, allowing substrate binding, phosphorylation, and downstream signaling. Finding a membrane-embedded ligand (diacylglycerol) by diffusion from the cytosol is a low-probability event, and, in the case of conventional PKCs, nature has chosen a clever mechanism to increase the efficiency of this. Binding of Ca^{2+} to the C2 domain essentially pretargets conventional PKC to the membrane (primarily plasma membrane, where the C2 domain binds phosphatidylinositol-bis-phosphate (PIP_2)), where it can now initiate a much more effective search for its membrane-embedded ligand, diacylglycerol. As a consequence, conventional PKCs translocate to membranes approximately one order of magnitude faster than novel PKC isozymes, which do not have the advantage of pretargeting by a Ca^{2+}-responsive C2 domain. Nonetheless, novel isozymes are able to respond to diacylglycerol because their C1b domain is tuned to be an order-of-magnitude more responsive to diacylglycerol; these isozymes preferentially bind Golgi, which is unusually rich in diacylglycerol.

The advent of fluorescent methodologies has allowed imaging of PKC translocation and activity in real time in living cells (**Fig. 2**) (Gallegos and Newton, 2008). Notably, targeted reporters have allowed the visualization of kinase translocation, activity, and second messenger levels at precise intracellular locations, from specific membranes to protein scaffolds. In general, PKC translocation and activity mirror the generation of its second messengers. For example, histamine stimulation of HeLa cells results in oscillations in PKC substrate phosphorylation that are phase-locked with Ca^{2+} oscillations. Conventional PKC isozymes translocate rapidly to plasma membrane with kinetics mirroring Ca^{2+} elevation; diacylglycerol retains these isozymes at the plasma membrane and their activity here decays in concert with diacylglycerol. Novel isozymes translocate more slowly to Golgi membranes, where their activity is sustained because of sustained diacylglycerol at the Golgi.

PKC Scaffolds

Correct subcellular location is essential for normal signaling by PKC. An abundance of scaffold proteins that tether PKC near its substrates, activators, and regulatory proteins, such as phosphatases, has been described, most notably receptors for activated C kinases. The importance of correct subcellular location is perhaps best illustrated in the *Drosophila* visual cascade, where mutants

lacking the PKC-binding scaffold, InaD, are defective in visual transduction because components of the signaling cascade are mislocalized.

PKC Downregulation

Prolonged activation of PKC by treatment of cells with phorbol esters results in degradation of PKC, a phenomenon referred to as downregulation. In fact, prolonged treatment of cells with phorbol esters was a commonly used approach to deplete cells of all except atypical PKCs (these are resistant to phorbol ester-dependent downregulation because they do not bind phorbol esters). The first step in downregulation is the dephosphorylation of the hydrophobic motif site by the PH-domain leucine-rich repeat protein phosphatase (PHLPP), an event that shunts PKC to a detergent-insoluble fraction where it is further dephosphorylated, ubiquitinated, and degraded via a proteosomal pathway. This phosphatase is also associated with newly-synthesized PKC and provides a quality control pathway to ensure that only PKC that is able to be properly autoinhibited is "matured" and enters the pool of signaling-competent enzyme. For example, mutations that impair autoinhibition will generally prevent accumulation of the mutant PKC in cells because PHLPP prevents retention of phosphate on the hydrophobic motif, triggering down-regulation.

PKC Signaling

The identification of PKC as the "receptor" for the tumor promoting phorbol esters led to the dogma that PKC is an oncogene (Griner and Kazanietz, 2007). However, three decades of cancer clinical trials using inhibitors of PKC were ineffective and in some cases worsened patient outcome. Recent analysis of cancer-associated mutations in tumors from patients revealed that PKC function is generally lost in cancer, redefining PKC as a tumor suppressor (Antal *et al.*, 2015). It is noteworthy that in the skin carcinogenesis models for tumor promotion, repeated phorbol ester applications likely causes the degradation and loss of PKC. While there are likely to be context-dependent exceptions, it is now becoming clear that high levels of PKC protein are associated with increased survival in many cancers, including pancreatic and colon cancer.

Whereas loss-of-function mutations are associated with cancer, a number of germline gain-of-function mutations have been identified in neurodegenerative disease. Mutations in the conventional PKCα that enhance its activity are associated with Alzheimer's Disease, and mutations in PKCγ that enhance activity are associated with Spinocerebellar Ataxia. These mutations evade quality control degradation, allowing enhanced activity without altering steady-state levels.

The amplitude of the PKC signal depends on (1) the cellular levels of PKC, which are controlled by phosphorylation and dephosphorylation, (2) the acute activity of PKC, which is controlled by second messengers, and (3) the subcellular localization of PKC, which is controlled not only by membrane interactions, but also by protein scaffolds. Each mechanism is precisely controlled. Dysregulation at any step results in pathophysiological states, with reduced function associated generally with cancer and enhanced function associated with degenerative disease.

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