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Protein Kinase C (Prkc)

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Historical Background

Protein kinase C was discovered in the late 1970s by Yasutomi Nishizuka and his team at Kobe University, Japan. They had originally purified a kinase that required only Mg^{2+} for activity, so they named it protein kinase M (PKM). It soon became apparent to the group that PKM was a proteolytic product of a larger enzyme whose kinase activity needed to be unmasked by cofactors. They named the parent enzyme protein kinase C because a Ca^{2+}-dependent protease cleaved it to release the unregulated kinase moiety they had initially purified. The subsequent identification of diacylglycerol as the key cofactor provided an explanation for how lipid hydrolysis, discovered 25 years earlier to be triggered by stimuli such as acetylcholine, couples to intracellular signaling pathways. But the discovery that shot PKC into the limelight was its specific binding to, and activation by, tumor-promoting phorbol esters, a discovery that was facilitated by the synthesis of relatively water-soluble phorbol esters by Peter Blumberg and his team. With tools to now study the enzyme, the field exploded with the identification of substrates of PKC and cellular functions impacted by phorbol ester treatment of cells. Extensive studies in the past 30 years have unveiled the exquisite details on the regulation of the enzyme and identified a central role in maintaining cellular homeostasis (see reviews Griner and Kazanietz 2007; Newton 2004; Nishizuka 1992; Rosse et al. 2010).

Protein Kinase C Family

There are nine isozymes of mammalian PKC classified into three subfamilies based on their cofactor dependence (Fig. 1): conventional PKCs (α, β, γ) are activated by diacylglycerol and Ca^{2+}, novel PKCs (δ, ε, η, θ) are activated by diacylglycerol, and atypical PKCs (ι, ζ) are regulated by protein scaffolds. All PKCs share a common architecture of an N-terminal regulatory moiety that constrains the catalytic activity of a C-terminal kinase domain. This is achieved by an autoinhibitory pseudosubstrate segment that occupies the substrate-binding cavity in the inactive conformation. The pseudosubstrate is immediately followed by a C1 domain, which occurs as two tandem C1 domains (C1A and C1B) in all but the atypical PKCs. The C1 domain serves as a diacylglycerol sensor in the conventional and novel PKCs, but not in the atypical PKCs because their C1 domain lacks one face of the ligand-binding pocket. The stoichiometry of binding of diacylglycerol, or their functional analogues, the phorbol esters, is one mole ligand per mole PKC,

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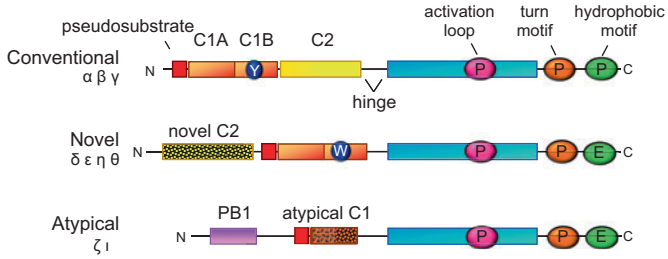


Second messengers		Pharmacological tools			
		activator	inhibitors		
DG	Ca^{2+}	phorbol esters	Gö6983	Gö6976	PZ09
+	+	+++	+	+	–
++	–	+++	+	–	–
–	–	–	–	–	+

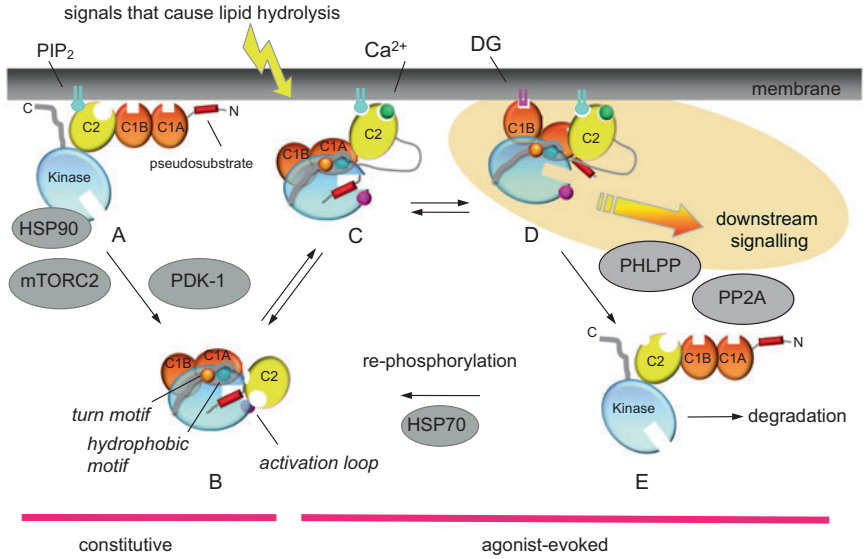
Protein Kinase C (Prkc), Fig. 1 Primary structure showing the domain composition of protein kinase C family members. The N-terminal regulatory moiety contains the autoinhibitory pseudosubstrate segment (*red*), the tandem diacylglycerol-binding C1 domains (*orange*), and the Ca^{2+}-binding C2 domain (*yellow*). The C2 domain in novel PKCs and the C1 domain in atypical PKCs are non-ligand-binding variants (*mottled*). The C1B domain of novel PKCs has a Trp at the position where conventional PKCs have a Tyr, a change that increases the affinity for diacylglycerol by two orders of magnitude. Atypical PKCs have a PB1 domain (*purple*) that mediates binding to protein scaffolds. The C-terminal kinase moiety contains the catalytic domain that has a priming phosphorylation site by PDK-1 (*pink*) and a C-terminal tail that is phosphorylated at the turn motif (*orange*) and hydrophobic motif (*green*); atypical PKCs have a Glu at the phosphoacceptor site of the hydrophobic motif. The second messenger sensitivity (diacylglycerol (*DG*) and Ca^{2+}), as well as sensitivity to pharmacological tools, is shown on the *right*

revealing that only one of the C1 domains binds ligand in the context of the full-length protein. A single amino acid difference from Tyr to Trp in the C1B domain increases the affinity of novel PKCs for diacyglycerol by two orders of magnitude compared to conventional PKCs. This allows novel PKCs to respond to agonist-evoked increases in diacylglycerol alone, without the need for increases in intracellular Ca^{2+}. Conventional PKCs also have a Ca^{2+}-sensing C2 domain which targets these PKCs to the plasma membrane via a recognition motif for phosphatidylinositol 4,5-bisphosphate (PIP_2), a lipid localized to plasma membrane. Although novel PKCs have a C2 domain, it lacks key Asp residues that coordinate Ca^{2+} and thus is not a Ca^{2+} sensor. Atypical PKCs, which respond to neither Ca^{2+} nor diacyglycerol, have a PB1 protein interaction domain that regulates their activity and subcellular location. As with most signaling enzymes, the cellular levels of PKCs are relatively low: quantitative analysis of the Hela proteome revealed that the cellular concentrations of individual PKCs detected in the study range from about 1 nM (PKCε) to 100 nM (PKCι) in the cell (Hein et al. 2015).

Regulation of Protein Kinase C

Phosphorylation

Conventional PKCs are processed by a series of ordered phosphorylations and ordered conformational transitions to yield a signaling-competent enzyme that is maintained in an autoinhibited conformation until the correct second messengers are present (Fig. 2). Studies with PKCβII have revealed that newly synthesized enzyme adopts an open conformation in which the pseudosubstrate and membrane-targeting modules are exposed. The molecular chaperone Hsp90 binds to this newly synthesized PKC via a conserved PXXP motif in the kinase domain, an interaction that stabilizes PKC and allows it to become phosphorylated and converted into an autoinhibited and stable species. The first phosphorylation is catalyzed by the phosphoinositide-dependent kinase, PDK-1, which binds the C-terminus of newly synthesized PKC and phosphorylates the activation loop, a segment near the entrance to the active site, in a reaction that is not regulated by phosphoinositides; this phosphorylation triggers two tightly coupled phosphorylations at two C-terminal sites, the turn motif (containing the



Protein Kinase C (Prkc), Fig. 2 Regulation of conventional PKC. Newly-synthesized conventional PKC is in a membrane-associated, open conformation (*A*); it is phosphorylated at the activation loop (*magenta circle*) by PDK-1 and two C-terminal sites (*orange and green circles*) by autophosphorylation, events that allow PKC to adopt a closed conformation in which the C2 domain (*yellow*) clamps the autoinhibitory pseudosubstrate in the active site (*B*); this autoinhibited PKC localizes to the cytosol. In response to agonists that promote PIP_2 hydrolysis, Ca^{2+} binds the C2 domain, and the Ca^{2+}-bound PKC is recruited to the plasma membrane (*C*), where it binds the membrane-embedded ligand, DG, resulting in the release of the pseudosubstrate from the substrate-binding cavity, thereby activating PKC (*D*). Mechanisms to loosen autoinhibitory constraints will enhance PKC signaling. When second messenger levels drop, PKC is released from the membrane and regains the autoinhibited conformation. However, prolonged membrane binding, as occurs upon treatment of cells with phorbol esters (which are not readily metabolized) results in the dephosphorylation and degradation of PKC (*E*). Note that the molecular chaperone Hsp70 can sustain the signaling lifetime of PKC by binding dephosphorylated PKC and allowing it to become rephosphorylated and reenter the pool of signaling-competent enzyme. Indicated in *gray* are key regulators of PKC discussed in the text

segment LTP) and the hydrophobic motif (with the consensus motif FXXFSF/Y). The kinase complex mTORC2 is required for the maturation of PKC, but the mechanism of action has not been elucidated. The fully-phosphorylated enzyme undergoes conformational transitions to adopt an autoinhibited conformation in which the pseudosubstrate is now tucked in the substrate-binding cavity, and the C1 and C2 domains become masked. The crystal structure of full-length PKCβII (Leonard et al. 2011) revealed an additional function of the C2 domain in clamping over the kinase domain of autoinhibited PKC, locking the pseudosubstrate segment in the substrate-binding cavity and ensuring minimal signaling in the absence of activators (Antal et al. 2015a). Disruption of any of the mechanisms that allow phosphorylation, such as disruptiong the binding of Hsp90, inhibiting mTORC2, removing PDK-1, or preventing autophosphorylation with kinase-inactive constructs of PKC, results in the degradation of PKC, and thus enzyme does not accumulate in cells. PKC matured by these phosphorylation events is the species that accounts for almost all the PKC in the cell, and it is this species that is activated by lipid hydrolysis and transduces signals.

Novel PKCs are all phosphorylated by PDK-1 at the activation loop, but only some depend on mTORC2 for processing phosphorylations. Notably, PKCδ, PKCη, and PKCθ do not require mTORC2 for processing and stability, but PKCε does (Facchinetti et al. 2008; Ikenoue et al. 2008). PKCδ is unusual in that it is the

only PKC reported to be active when expressed in bacteria, albeit with low activity (Stempka et al. 1997), and its is also regulated by Tyr phosphorylation (Humphries et al. 2008; Steinberg 2004).

Atypical PKCs are also constitutively phosphorylated, but their mechanism of phosphorylation differs in a few key aspects (Tobias et al. 2016). First, atypical PKCs, like their related cousin Akt, are co-translationally phosphorylated at the turn motif by ribosome-associated mTORC2. This phosphorylation at the turn motif is followed by constitutive phosphorylation at the activation loop by PDK-1. These are the only processing phosphorylations on atypical PKCs as they have a Glu at the position of hydrophobic motif phosphorylation site of the other PKCs. Thus, like the conventional and novel PKCs, atypical PKCs are constitutively phosphorylated, but the mechanisms controlling the phosphorylations differ slightly.

Activation

PKC that has been processed by phosphorylation is a cytosolic enzyme that is autoinhibited but poised to respond to second messengers. For conventional PKCs, binding of Ca^{2+} to the C2 domain recruits these isozymes to the plasma membrane. This membrane translocation is a hallmark of PKCs and is accompanied by unmasking of the hinge segment that links the kinase domain and the regulatory moiety; biochemical studies have revealed that the proteolytic sensitivity of this region increases by two orders of magnitude upon Ca^{2+}-dependent membrane binding. Once at the membrane, PKC binds its membrane-embedded ligand, diacylglycerol, prompting a second conformational change that expels the pseudosubstrate from the substrate-binding cavity. This allows substrate phosphorylation and downstream signaling. Pioneering technologies by Roger Tsien and colleagues led to the development of fluorescence resonance energy transfer (FRET) reporters to measure PKC signaling in real time in live cells at specific locations (Violin et al. 2003). Such studies have revealed that in response to agonists that promote phospholipid hydrolysis, conventional PKCs translocate primarily to the plasma membrane with kinetics that mirror the rise in intracellular Ca^{2+}. Their return to the cytosol mirrors the decay kinetics of plasma membrane diacylglycerol. In contrast, novel PKCs translocate primarily to Golgi membranes, with the kinetics of translocation mirroring the kinetics of diacylglycerol increases at this location. The sustained elevation of diacylglycerol at the Golgi results in sustained signaling of novel PKCs.

Atypical PKCs are regulated by protein scaffolds that not only position these PKCs near their substrates but also relieve autoinhibitory constraints by tethering the pseudosubstrate away from the substrate-binding cavity. These PKCs have a particularly slow catalytic rate, so their colocalization with substrates ensures efficient phosphorylation.

Small molecule inhibitors that are selective for PKC and useful in cellular studies are the bisindolylmaleimides Gö6983, which inhibits conventional and novel PKCs, and Gö6976, which inhibits conventional PKCs (but also protein kinase D) (Wu-Zhang and Newton 2013). Note that PKCs bound to protein scaffolds are generally refractory to active site inhibitors but not BisIV, an uncompetitive inhibitor with respect to substrates (Hoshi et al. 2010). PZ09 is a small molecule inhibitor which effectively inhibits atypical PKCs (Tobias and Newton 2016; Trujillo et al. 2009).

Downregulation

The active, membrane-bound conformation of PKC is sensitive to dephosphorylation, and prolonged activation, as occurs upon treatment of cells with phorbol esters, results in the dephosphorylation and degradation of PKC, a process referred to as downregulation (Hansra et al. 1999). Indeed, overnight treatment with phorbol esters was a commonly employed procedure to deplete cells of PKC before the advent of siRNA and gene-editing technologies. Studies with the conventional PKCβII have revealed that the first step in the downregulation is dephosphorylation at the hydrophobic motif, a reaction that is catalyzed by the PH domain leucine-rich repeat protein

phosphatase (PHLPP) (Gao et al. 2008). This destabilizes the kinase domain, promoting subsequent dephosphorylation at the turn motif and activation loop by PP2A phosphatases, ubiquitination, and proteasomal degradation. Note that the molecular chaperone Hsp70 binds the dephosphorylated turn motif, an event that stabilizes PKC and promotes its rephosphorylation and reentry into the pool of signaling-competent enzyme. Atypical PKCs are neither downregulated by phorbol esters (they do not have a ligand-binding C1 domain) nor are they dephosphorylated by PHLPP (they have a Glu at the hydrophobic motif).

Function of Protein Kinase C

The synthesis of phorbol dibutyrate provided a tool to identify PKC substrates and an abundance of proteins whose phosphorylation changes upon treatment of cells with phorbol esters have been annotated in the literature. Given the expression of multiple isozymes in a single cell type and their general activation by phorbol esters, defining the precise biological role of PKC has been a challenge. In fact, for 30+ years, PKCs were considered to be oncogenes. Yet clinical trials for cancer using PKC inhibitors not only proved unsuccessful but in some cases worsened patient outcome. It is thus not surprising that a recent analysis of approximately 50 of the 500+ somatic mutations identified in diverse human cancers revealed that two third were loss of function, and none were activating (Antal et al. 2015b). This suggests that a major function of PKCs is to suppress survival signaling, such that loss of PKC would confer a survival advantage to cells. In this regard, many of the substrates of PKC are proto-oncogenes that are inactivated following phosphorylation by PKC. Notably, the EGF receptor is phosphorylated and downregulated by PKC, and K-Ras is phosphorylated and inactivated by PKC. Indeed, a recent study suggested that enhancing the activity of PKC by a weak phorbol ester, prostratin, suppressed K-Ras-mediated oncogenesis in a mouse model (Wang et al. 2015). Thus, mounting evidence supports a role for PKC in suppressing, rather than enhancing, oncogenic signaling.

A major reason why PKCs were considered oncogenes is because they are acutely activated by phorbol esters, potent tumor promoters: phorbol esters are not themselves mutagenic or carcinogenic, but promote tumor growth when repetitively applied to the skin of mice pretreated with a subthreshold amount of a carcinogen (Griner and Kazanietz 2007). However, as noted above, prolonged treatment with phorbol esters results in the loss of PKC. Thus, the chronic downregulation of PKC, rather than its acute activation, may contribute to the tumor-promoting properties of phorbol esters.

Contrasting with the loss-of-function mutations in PKCs in cancer, germline mutations that enhance the activity of PKC are associated with degenerative diseases: activity-enhancing mutations in PKCγ cause spinocerebellar ataxia (Verbeek et al. 2008) and ones in PKCα cosegregate with affected individuals in families with Alzheimer's disease (Alfonso et al. 2016). Thus, cellular PKC activity must be exquisitely balanced to avoid pathologies from either too little or too much activity.

Summary

The amplitude of the protein kinase C signal depends on (1) the cellular levels of protein kinase C, which are in part controlled by *phosphorylation* and *dephosphorylation*; (2) the acute activity of protein kinase C, which is controlled by *second messengers* (diacylglycerol and Ca^{2+}) and *autoinhibitory contacts*; and (3) the subcellular localization of protein kinase C, which is controlled not only by *membrane interactions* but also by *protein scaffold interactions*. Each mechanism is precisely controlled. Dysregulation at any step results in pathophysiological states, with not enough activity associated with cancer and too much activity associated with degenerative diseases. This underscores the importance of precisely regulating the signaling output of PKC in cells for normal homeostasis.

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