Protein kinase C: poised to signal

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Newton AC. Protein kinase C: poised to signal. Am J Physiol Endocrinol Metab 298: E395–E402, 2010. First published November 24, 2009; doi:10.1152/ajpendo.00477.2009.—Nested at the tip of a branch of the kinome, protein kinase C (PKC) family members are poised to transduce signals emanating from the cell surface. Cell membranes provide the platform for PKC function, supporting the maturation of PKC through phosphorylation, its allosteric activation by binding specific lipids, and, ultimately, promoting the downregulation of the enzyme. These regulatory mechanisms precisely control the level of signaling-competent PKC in the cell. Disruption of this regulation results in pathophysiological states, most notably cancer, where PKC levels are often grossly altered. This review introduces the PKC family and then focuses on recent advances in understanding the cellular regulation of its diacylglycerol-regulated members.

calcium; diacylglycerol; phorbol esters

THE FAMILY OF PKC ISOZYMES transduces the myriad of signals resulting from receptor-mediated hydrolysis of phospholipids, playing critical roles in diverse cellular functions. The discovery in the 1980s that they are the receptors for the potent tumor promoting phorbol esters, coupled with the discovery that they mediate signaling by the lipid second messenger diacylglycerol, secured a center stage position for this family of enzymes in cellular regulation (6, 66). Family members, themselves, are regulated by precise mechanisms that control their structure, function, and subcellular localization. This review discusses our current understanding of the mechanisms controlling protein kinase C signaling.

The PKC Family

The PKC family occupies the tip of a branch of the AGC kinases from which the related kinases protein kinase N (PKN), Akt/PKB, p70 S6 kinase, and the phosphoinositide-dependent kinase-1 (PKD-1) diverge (Fig. 1A). The 10 members that populate the mammalian family evolved from the single PKC in Saccharomyces cerevisiae, PKC1 (57, 98), and are grouped into three classes based on their domain composition (Fig. 1B) (71). This domain composition, in turn, dictates the cofactor regulation of the classes (Fig. 1C). At the very tip of the branch are the four conventional isozymes: PKCα, the first isoform cloned (76); the alternatively spliced PKCβ1 and PKCβII (which differ in the last 43 amino acids) and PKCy; next are the four novel isozymes, PKCδ, -ε, -η, and -θ; closest to the point of divergence are the atypical isozymes PKCζ and PKCe (human; murine isoform is PKCA).

All family members share the same architecture: a carboxy-terminal kinase domain linked by a flexible hinge segment to an amino-terminal region containing regulatory modules (Fig. 1B) (69, 77). These regulatory modules confer sensitivity to the second messengers diacylglycerol (C1 domain) or Ca2+ (C2 domain), although, importantly, some isoforms have variants of these modules that do not bind ligand (8, 20).

Conventional isoforms contain tandem C1 domains that bind diacylglycerol and a C2 domain that binds anionic lipids in a Ca2+-dependent manner. The small globular C1 domain is also the binding site for the potent tumor-promoting phorbol esters (43, 100), which bind competitively with respect to diacylglycerol (87). The C1 domain stereospecifically binds the anionic phospholipid phosphatidylserine (46, 70). Pioneering work by Blumberg and colleagues, who developed lipophilic analogs of phorbol esters that allowed, for the first time, demonstration of specific and saturable binding of phorbol esters to cells (22), identified the stoichiometry of phorbol ester binding as 1 mol ligand/mol PKC (53); for most isoforms, it is the C1b domain that is the relevant binding module in the context of the full-length protein (49, 80). The C2 domain of conventional PKC isoforms binds anionic phospholipids with modest, but not stereospecific, selectivity for phosphatidylserine (16, 19, 46, 61). More significantly, it has a strong preference for phosphatidylinositol-4,5-bisphosphate (PIP2), and it is this preference for PIP2, which is mediated by a basic patch distal to the Ca2+ binding site (Fig. 1B; oval with + + indicated in the C2 domain of conventional PKC isoforms), that selectively targets conventional PKC isoforms to the plasma membrane (20, 28, 59).

Novel isoforms also contain tandem C1 domains that bind diacylglycerol; these isoforms contain a variant of the C2 domain (novel C2) that lacks key residues that coordinate Ca2+ and, as a result, the novel isoforms are not sensitive to Ca2+. These isoforms are thus regulated only by diacylglycerol. Because these isoforms are regulated by only one membrane-targeting module, their affinity for diacylglycerol is two orders of magnitude higher than that for the conventional PKC isoforms (35). A single residue in the C1b domain tunes the module from a high-affinity diacylglycerol sensor in novel isoforms (which have a Trp at a conserved position on the membrane proximal moiety of the C1b domain) to a low-
affinity diacylglycerol sensor for conventional isozymes (which have a Tyr at the equivalent position on their C1b domain) (23). This increased affinity allows novel PKC isozymes to respond to agonist-evoked increases in diacylglycerol. Conventional PKC isozymes depend on membrane recruitment by the Ca\textsuperscript{2+}-regulated C2 domain to sense agonist-evoked diacylglycerol. Atypical isozymes contain a variant of the C1 domain (atypical C1) with an impaired ligand-binding pocket that binds neither diacylglycerol nor phorbol esters (48, 81). Nor are they regulated by Ca\textsuperscript{2+}; rather, protein-protein interactions provide the major driving force for controlling the function of these isozymes in cells. To this end, they contain a PB1 domain (Fig. 1B) involved in protein interactions (54) as well as a carboxyl-terminal PDZ ligand.

All isozymes have a conserved carboxyl-terminal tail (CT) that serves as a phosphorylation-dependent docking site for key regulatory molecules (Fig. 1B; CT; phosphorylation sites indicated by ovals). In addition, all isozymes have an autoinhibitory pseudosubstrate sequence (Fig. 1B, green rectangle) that maintains PKC in an inactive state by occupying the substrate-binding cavity. Although the structure of full-length PKC has remained refractory to elucidation, structure-function studies reveal that many other contacts maintain the enzyme in a “closed” state in the absence of membrane binding (59, 90).

The past few years have seen the determination of the structure of the kinase domain of several PKC isozymes [PKC\textbeta II, PKC\textepsilon, and PKC\textgamma (38, 62, 99)]. Particularly noteworthy in the crystal structure of the PKC\textbeta II kinase domain is the clear identification of the three key priming phosphorylations discussed below: the activation loop and the two sites on the CT, which is well ordered in this structure (Fig. 2).

**Maturation of PKC**

PKC isozymes are matured by a series of ordered, tightly coupled, and constitutive phosphorylations that are essential for the stability and catalytic competence of the enzyme (69, 78). Recent studies have identified two new players in this maturation: heat shock protein-90 (HSP90), whose interaction with a specific motif on PKC is essential to allow phosphorylations to occur (36), and the mammalian target of rapamycin (mTOR) complex 2 (mTORC2), a structure comprised of the kinase mTOR, Sin1, Rictor, and mLST8, whose integrity is required to allow the priming phosphorylations (39, 44).

**Regulation by HSP90.** The earliest identified step in the maturation of conventional and novel PKC is the binding of the chaperone HSP90 and the co-chaperone Cdc37 to a molecular clamp in the kinase domain formed by a conserved PXXP motif (Fig. 2, Pro in green) in the CT with a conserved Tyr on
isozymes. The activity of PDK-1 toward conventional PKC isozymes has been shown to be independent of phosphoinositides (88). Rather, phosphorylation is controlled by the conformation of PKC: newly synthesized PKC is in an open conformation in which the pseudosubstrate is removed from the substrate-binding cavity, thus unmasking the activation loop site to allow phosphorylation by PDK-1 (Fig. 3) (25). The maturation of PKC requires priming phosphorylation by PDK-1, but once the enzyme is phosphorylated at the two CT sites, phosphorylation at this site, at least for PKCβII, becomes dispensable (25). Indeed, this site is dephosphorylated in a serum-sensitive manner such that only about one-half of the pool of conventional PKC in cells cultured in serum is phosphorylated on the activation loop, yet is quantitatively phosphorylated at the two CT sites (50, 88).

Phosphorylation by PDK-1 is likely the first phosphorylation event in the processing of PKC by phosphorylation. First, as noted above, replacement of the Thr at the activation loop of conventional PKC isozymes with neutral, nonphosphorylable residues (Ala or Val) prevents the subsequent phosphorylations on the CT sites, indicating that phosphorylation of the activation loop is necessary to allow CT phosphorylation (15, 74). Second, locking negative charge on either of the COOH-terminal sites by Glu substitution does not significantly alter the rate of processing of PKCβII to the fully phosphorylated species, indicating that phosphorylation of the CT sites is not rate limiting, as would be expected if these were the first sites modified (88). Taken together, the most likely mechanism for the PDK-1 step is that it is first and necessary for the processing of PKC to the fully phosphorylated form. Because perturbation of the PDK-1 step prevents the maturation of PKC by phosphorylation, thus resulting in species of PKC that are highly susceptible to degradation, this step plays a critical role in controlling cellular levels of PKC.

**TURN MOTIF.** Structural analysis reveals that phosphorylation of the turn motif stabilizes the structure of mature PKC by anchoring the carboxyl-terminal tail on the upper lobe of the kinase (41). Specifically, the phosphate on the turn motif binds a cluster of basic residues in a pocket above the ATP-binding Gly loop to stabilize the active conformation of the kinase. Curiously, mutation of the turn motif residue in PKCβII to Ala results in compensating phosphorylations that accompany the processing of PKC to a functional species; however, mutation of adjacent Thr to Ala prevents these compensatory phosphorylations and abolishes the processing of PKCβII (27). These data suggest that the precise presence of phosphate on the actual turn motif Thr is dispensable, supported by the finding that similar mutation in PKCo allows maturation of PKCo, albeit to a more thermally-labile and phosphatase-sensitive species (9).

It has recently been shown that phosphorylation of the turn motif depends on the mTORC2 complex (29, 44, 45). Importantly, PKC cannot be processed by phosphorylation in cells lacking this complex, and, because the unphosphorylated species is unstable, it is degraded (39, 44). Whether mTORC2 controls the phosphorylation of this site indirectly, for example by chaperoning or positioning newly synthesized PKC for processing by phosphorylation, or by activating another upstream kinase for this site, or whether it directly phosphorylates PKC is unclear. It is noteworthy that mTORC2 is not able to phosphorylate PKC in vitro at the turn motif (44).
Cells lacking mTORC2 have grossly reduced levels of PKC, consistent with the essential role of priming phosphorylations in stabilizing PKC. Thus, steps that interfere with the ability of mTORC2 to control turn motif phosphorylation are another Achilles' heel in PKC regulation.

HYDROPHOBIC MOTIF. Kinetic analyses with pure protein have revealed that PKC autophosphorylates by an intramolecular reaction at the hydrophobic motif (7). Whether this is the mechanism of regulation in cells has been difficult to resolve. This site can be phosphorylated in vitro by a number of kinases, including mTORC2 (44). Modulation of this site by the TOR kinase was first reported by Parker et al. (101), who showed that retention of phosphate on the hydrophobic motif of PKCβ is sensitive to the inhibitor rapamycin. More recent studies have established that the hydrophobic motif is not phosphorylated in mTORC2-deficient cells. This may be because TOR directly controls the phosphorylation of this site or because a prerequisite event to allow hydrophobic motif phosphorylation has not taken place (27).

The phosphorylation of the hydrophobic motif is also controlled by the interaction of HSP90 with the PXXP clamp described; inhibitors of HSP90 slow the phosphorylation of this site but not the turn motif site (36). It is noteworthy that PKCβ is not phosphorylated at the hydrophobic motif (constitutive negative charge at this position), nor does its PXXP motif control HSP90 binding. Thus, HSP90 facilitates the phosphorylation of the hydrophobic motif of conventional and novel PKC isozymes by its interaction with the PXXP clamp.

Stabilization of phosphorylation by active site inhibitors. Constructs of PKC that are catalytically inactive because an essential Lys in the active site that coordinates the α/β phosphates of ATP has been mutated (72) are not processed by phosphorylation (13). It has recently been shown that the binding of active site inhibitors to these constructs stabilizes the phosphorylation of the active site also observed for PKB (73). This suggests that occupancy of the active site by inhibitors stabilizes a conformation that either promotes the phosphorylation of PKC or protects the phosphorylated enzyme from dephosphorylation. It is noteworthy that occupancy of the active site with the pseudo-substrate (i.e., in the inactive conformation of PKC) (24) or with peptide substrates protects pure PKC from dephosphorylation (25). Consistent with active site occupancy “freezing” the CT in a conformation that masks the phosphorylation sites, structural studies with PKA have shown that the CT is highly flexible in the apostructure and highly ordered when active site inhibitors are bound (1, 65). Note the structure of the kinase domain of PKCβII that shows that a highly ordered CT was obtained with
bound active-site inhibitor (38). The ability of active site inhibitors to allow accumulation of phosphate on kinase-dead constructs of PKC supports the possibility that other kinases, perhaps TORC2, modify the hydrophobic motif in cells (13); however, mechanistic conclusions await the dissection of phosphorylation versus dephosphorylation steps in promoting phosphate retention.

**Regulation by Lipid Second Messengers**

The activity of mature conventional and novel PKC isozymes is acutely regulated by binding diacylglycerol, an event that is assisted by Ca\(^{2+}\) for the conventional PKC isozymes (reviewed recently in Refs. 6, 33, 67, 89). For conventional PKC isozymes, the mature enzyme localizes primarily to the cytosol, where it is likely maintained in precise microenvironments by scaffold interactions (86). Agonist-evoked hydrolysis of PIP\(_2\) generates two key second messengers: Ca\(^{2+}\) and diacylglycerol. Binding of Ca\(^{2+}\) to the C2 domain pretargets PKC to the plasma membrane, where it binds anionic phospholipids, with selectivity for PIP\(_2\). Once engaged on the membrane, the C1 domain binds its membrane-embedded ligand diacylglycerol, an interaction that is enhanced by stereospecific binding to phosphatidylserine. The coordinated engagement of both the C1 and C2 domains on the membrane provides the energy to release the autoinhibitory pseudo-substrate sequence from the substrate-binding cavity, allowing substrate phosphorylation. As noted above, novel PKC isozymes are able to respond to agonist-evoked changes in diacylglycerol because their C1b domain has a two-orders-of-magnitude higher affinity for diacylglycerol-containing membranes than the C1b domain of conventional isozymes (35). Note that the higher affinity of novel isozymes for diacylglycerol results in significant basal localization to membranes enriched in diacylglycerol, notably Golgi (14).

The advent of imaging technologies to visualize the generation of second messengers, the subcellular location of PKC, and the activity of PKC at precise cellular locations has allowed much insight into the spatiotemporal dynamics of PKC signaling (12, 32, 33, 96). Fluorescence energy transfer (FRET)-based activity reporters have revealed that Ca\(^{2+}\) oscillations, with or without diacylglycerol oscillations, drive oscillations of the activity of conventional PKC (5, 93, 97). They have also revealed that oscillatory activity does not necessarily require oscillatory membrane translocation: oscillations in Ca\(^{2+}\), under conditions were diacylglycerol levels are sustained and thus retain PKC at the plasma membrane, result in oscillations of activity (97). Live-cell imaging studies have also established that there are different “signatures” of PKC activity at defined microenvironments in cells (32). That is, the level of basal signaling and rate, magnitude, and duration of agonist-evoked signaling vary depending on cellular location and isozyme. Most notably, rapid rises in intracellular Ca\(^{2+}\) drive rapid activation of conventional PKC isozymes at the plasma membrane, where they are likely targeted via unique interactions of their C2 domain with PIP\(_2\), a lipid enriched at the plasma membrane (59). Golgi membranes have relatively high levels of diacylglycerol, mediating a “basal” interaction of novel PKC isozymes at the Golgi (14). Agonist-evoked increases in diacylglycerol are highly sustained at the Golgi, resulting in sustained activation of both novel and conventional PKC isozymes at Golgi. In contrast, diacylglycerol at the plasma membrane is transient, resulting in transient activation of conventional PKC isozymes at the plasma membrane. Use of FRET-based activity reporters has recently shown that naturally occurring mutations in PKC\(\gamma\) that are associated with spinocerebellar ataxia type 14 result in reduced signaling output in cells (95).

**Regulation by Scaffold Interactions**

The importance of protein scaffolds in coordinating PKC activity was first recognized by Mochly-Rosen and colleagues almost 20 years ago (63, 64). Her laboratory went on to identify and characterize a number of structurally unrelated scaffold proteins named receptors for activated C kinase (RACKs) that have in common the ability to bind specific PKC isozymes in a manner that relieves autoinhibition of the isozymes (83). Specifically, her laboratory identified stretches of sequence on scaffold proteins that resemble sequences in specific PKC isozymes and proposed that these sequences participate in intramolecular interactions that clamp PKC in an autoinhibited state. The sequence on the scaffold would compete for these intramolecular interactions, binding PKC and trapping it in an activated conformation. Thus, binding to such scaffolds not only localizes PKC but has the potential to sustain PKC signaling in the absence of second messenger binding. The use of peptides based on these sequences to disrupt the interaction of specific PKC isozymes with their cognate RACKs has been used to modulate the cellular function of PKC isozymes (18). Such peptides have been particularly valuable pharmacological tools to regulate the activity of PKC isozymes in heart disease (75). Note that there are scaffolds for all conformations and species of PKC, from never-phosphorylated, phosphorylated but inactive, phosphorylated and active (e.g., RACKs), to dephosphorylated PKC.

The demonstration by Zuker and colleagues over a decade ago, that disruption of the interaction between the eye-specific PKC in *Drosophila melanogaster* and the PDZ scaffold InaD impairs light signaling, continues to provide one of the most elegant demonstrations of the role of protein scaffolds in coordinating PKC activity (91). It is now clear that scaffold interactions are an integral part of PKC regulation, poising specific isozymes near key protein and lipid regulators and near substrates.

Misllocalization likely plays a role in pathophysiological states. Most strikingly, Joubert and coworkers (79, 94) identified a natural mutation in PKC\(\alpha\) (D294G in the hinge region) in human pituitary and thyroid tumors, which results in loss of selective targeting to cell-cell contacts. They then went on to show that the sequence around this mutation (GDE in wild-type enzyme) is a targeting sequence that drives PKC\(\alpha\) and PKC\(\varepsilon\) to cell-cell contacts (82). This sequence was recently shown to overlap with a 14-3-3 binding region that alternatively targets PKC\(\varepsilon\) from cell-cell contacts (via GDE motif) to cytosolic sequestration (via binding 14-3-3), where a new set of functions is unveiled (21). With respect to the latter, Parker and coworkers reported that PKC\(\varepsilon\) is phosphorylated at three sites in the hinge segment (see Fig. 1B), creating a binding site for 14-3-3 (85). This binding stabilizes an open conformation of PKC\(\varepsilon\) and promotes localized activity at the midbody during
cytokinesis. The ability to switch from cytosolic sequestration via 14-3-3 binding to cell–cell contact binding via GDE motif underscores the complex regulation of PKC function by protein interactions.

Downregulation

The life cycle of conventional and novel PKC isozymes is terminated by a process referred to as downregulation (40). In the absence of chronic stimulation, these PKC isozymes have a relatively long half-life (on the order of days for conventional PKC in cells grown in culture). However, sustained activation, as occurs upon treatment of cells with phorbol esters, results in the rapid degradation of PKC. In fact, phorbol ester treatment has been used as a mechanism to deplete cells of the phorbol ester-responsive isozymes, the conventional and novel PKC isozymes (42, 56). Note that atypical isozymes do not bind phorbol esters and are not downregulated in the same manner as their phorbol ester-binding counterparts.

In the inactive conformation, conventional and novel PKC isozymes are relatively resistant to dephosphorylation. However, when they are in the open, membrane-bound conformation, their sensitivity to dephosphorylation increases by two orders of magnitude (24). Thus, prolonged activation of PKC results in dephosphorylation, with the dephosphorylated species being unstable and shunted to degradation (see Fig. 3). The recently discovered PH domain leucine-rich repeat protein phosphatase [PHLPP; (11)] dephosphorylates the hydrophobic motif of conventional and novel PKC isozymes, an event that shunts them to a detergent-insoluble cell fraction, where they are dephosphorylated at the turn motif by an okadaic-sensitive phosphatase (34). The precise mechanisms for degradation remain to be elucidated, and it is likely that different isozymes will be controlled by unique “turn-off” mechanisms.

Summary

The past few years have seen the identification of new players that regulate the maturation, subcellular location, and downregulation of PKC. HSP90 and mTORC2 are key components of the maturation of PKC, and disruption of either interaction results in the inability of PKC to process into a stable and catalytically competent conformation. Conversely, PHLPP is a key component in the dephosphorylation step preceding downregulation of PKC, and inhibition of this dephosphorylation results in accumulation of PKC.

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REFERENCES


