The C2 domains of classical/conventional PKCs are specific PtdIns(4,5)P₂-sensing domains

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Abstract
The C2 domains of cPKCs [classical/conventional PKCs (protein kinase Cs)] bind to membranes in a Ca²⁺-dependent manner and thereby act as cellular Ca²⁺ effectors. Recent findings have demonstrated that the C2 domain of cPKCs interacts specifically with PtdIns(4,5)P₂ through its polybasic cluster located in the β3-β4-strands, this interaction being critical for the membrane localization of these enzymes in living cells. In addition, these C2 domains exhibit higher affinity to bind PtdIns(4,5)P₂ than any other polyphosphate phosphatidylinositol. It has also been shown that the presence of PtdIns(4,5)P₂ in model membranes decreases the Ca²⁺ concentration required for classical C2 domains to bind them. Overall, the studies reviewed here suggest a new mechanism of membrane docking by the C2 domains of cPKCs in which the local densities of phosphatidylserine and PtdIns(4,5)P₂ on the inner leaflet of the plasma membrane are sufficient to drive Ca²⁺-activated membrane docking during a physiological Ca²⁺ signal.

PKC (protein kinase C) is a large family of phospholipid-dependent serine/threonine kinases, which is activated by many extracellular signals and plays a critical role in several signalling pathways in the cell. The mammalian isoenzymes have been grouped into three subfamilies according to their enzymatic properties. The first group, called classical or conventional isoenzymes [cPKCs (classical/conventional PKCs)], includes PKCα, βI, βII and γ, all of which contain the conserved C1 and C2 domains in the regulatory region. These isoenzymes are regulated by Ca²⁺ and acidic phospholipids, which interact with the C2 domain, and by DAG (diacylglycerol), which interacts with the C1 domain. Recent studies have revealed that PtdIns(4,5)P₂ itself binds to a polybasic region in the C2 domain.

PtdIns(4,5)P₂ is a membrane phospholipid that regulates many important cellular processes. For a long time, it was supposed that the only role of PtdIns(4,5)P₂ was to act as a precursor of Ins(1,4,5)P₃ and DAG, leading to the release of Ca²⁺ from intracellular stores and the activation of many isoenzymes of the PKC family respectively. However, a myriad of recent studies has revealed additional signalling roles not only for PtdIns(4,5)P₂ itself but also for the whole family of phosphoinositides, which function as site-specific signals on membranes that recruit and/or activate proteins for the assembly of localized functional complexes.

C2 domains were initially described as the second of the four conserved functional domains found in the classical (or conventional) isoforms of mammalian Ca²⁺-dependent PKCs (cPKCs) [1]. Structurally, they share a common overall fold: a single compact Greek-key motif comprising eight antiparallel β-strands assembled in a β-sandwich architecture with flexible loops on top and at the bottom [2,3]. The main role of the C2 domain in cPKCs is to act as the Ca²⁺-activated membrane-targeting region [2,3]. This C2 domain displays two functional motifs. The first is the Ca²⁺-binding region, located in the flexible top loops, that binds two or three Ca²⁺ ions, depending on the isoenzyme, and also interacts with phosphatidylserine [4–6] (Figure 1). The second is a polybasic cluster, also called the lysine-rich cluster [2]. It is located in strands β3 and β4 (Figure 1), where it was found to be an additional binding site for anionic phospholipids when the three-dimensional structure of the domain bound to soluble phospholipids was determined [6,7].

The C2 domains of cPKCs bind preferentially to PtdIns(4,5)P₂ through their polybasic cluster
Extensive biochemical assays have been performed to assess the role of the polybasic cluster in the localization and activation of full-length PKCα. Thus site-directed mutagenesis studies performed for each motif shed light on their differential lipid affinities. Mutations that knocked-down the Ca²⁺-binding region abolished the Ca²⁺/phosphatidylserine binding of the C2 domain, but the ability to bind PtdIns(4,5)P₂-containing vesicles still persisted although with lower affinity [6,8,9]. On the contrary, mutations that knocked-down the polybasic cluster did not affect Ca²⁺/phosphatidylserine binding but completely abolished PtdIns(4,5)P₂ binding [6,8–10]. This suggests that Ca²⁺ is always needed to dock the domain to the membrane, while phosphatidylserine targets the domain by interacting
with the Ca\(^{2+}\)-binding region and PtdIns(4,5)P\(_2\) targets the domain by interacting with the polybasic cluster.

During a cytoplasmic Ca\(^{2+}\) signal, freely diffusing cPKCs are activated by Ca\(^{2+}\) binding to the C2 domain and then dock to the plasma membrane [11–14]. The resulting association brings the signalling domains of these proteins to the appropriate target membrane surface, leading to interaction with their effectors. The existence of these two distinct binding sites in C2 domains suggested that cPKCs may be functioning in a variety of ways so far not described, since these proteins could well be activated in a phosphatidylserine- and/or PtdIns(4,5)P\(_2\)-dependent manner. Owing to that, it was important to elucidate the molecular mechanism underlying C2 domain-target lipid recognition and membrane docking if we were to fully understand this signalling process.

In vivo cell studies showed that the polybasic cluster is essential for membrane docking in different cell models, independently of the level of DAG generated by PtdIns(4,5)P\(_2\) hydrolysis [9,10,13]. This suggests that the C2 domain of cPKCs binds Ca\(^{2+}\), which in turn induces protein translocation from the cytosol to the plasma membrane, where it recognizes both phosphatidyserine and PtdIns(4,5)P\(_2\) simultaneously, through the Ca\(^{2+}\)-binding region and polybasic cluster respectively. Studies regarding the orientation of the domain by EPR and X-ray diffraction also corroborated this premise since they showed that the domain docks in a nearly parallel orientation with respect to the plasma membrane surface [4,6,15]. In addition, PtdIns(4,5)P\(_2\) is highly charged (\(z = -4\)) and forms protuberances in the homogeneously distributed negative membrane formed by phosphatidyserine [16], thus permitting the dual interaction of the C2 domain with the two phospholipids (see model in Figure 2).

The specificity of the PtdIns(4,5)P\(_2\)-polybasic cluster interaction has recently been resolved. A wide variety of phosphoinositides were tested by in vitro FRET (fluorescence resonance energy transfer) experiments using small unilamellar vesicles containing a fluorescent phospholipid acting as an energy acceptor and the tryptophan residues of the C2 domain studied [\(\alpha, \beta\) and \(\gamma\)] acting as energy donors. The best binding affinity was obtained with PtdIns(4,5)P\(_2\)-containing vesicles, while the other double-phosphorylated phosphoinositides [PtdIns(3,5)P\(_2\) and PtdIns(3,4)P\(_2\)] induced very low binding affinities, in spite of the number of negative

Figure 1 | Overall structure of the C2 domain of PKCα
Ribbon diagram of the PKCα-C2 domain structure. The two functional motifs are shown: Ca\(^{2+}\)-binding region, formed by loops 1, 2 and 3 and located on top of the domain. This region is responsible for Ca\(^{2+}\) and phosphatidyserine binding. The second motif is labelled as polybasic and is formed by several lysine residues located in the \(\beta\)3- and \(\beta\)4-strands. \(\beta\) Strands are shown in green, \(\alpha\)-helices in red and loops in grey. Ca\(^{2+}\) is shown as yellow spheres. The residues mutated in the two regions are shown as a stick model with carbon in grey, nitrogen in blue and oxygen in red. The dotted line represents the membrane surface. The PDB code used was 1DSY.

Figure 2 | Schematic representation of the membrane-bound PKCα docked to the membrane surface
The model membrane corresponds to a POPC Molecular Dynamics simulation with co-ordinate files in ‘.pdb’ format (popc128a) [20]. The membrane is represented as a stick model with carbon in grey, nitrogen in blue and oxygen in red. The overall structure of the C2 domain of PKCα is represented as a cartoon model in purple (PDB code 1DSY). The four lysine residues located in the lysine-rich cluster are represented by a stick model with carbon in grey and nitrogen in blue. In the translocation model, Ca\(^{2+}\) (represented by yellow balls) is the driving force inducing localization of the C2 domain in the membrane by binding to the Ca\(^{2+}\)-binding region. There, Ca\(^{2+}\) acts as a bridge between two aspartate residues and the phosphatidyserine (PtdSer) in the membrane. In addition, the C2 domain interacts with PtdIns(4,5)P\(_2\) through the polybasic cluster, which is located in the \(\beta\)3-\(\beta\)4-strands, serving as a target that recruits PKCα to specific areas of the plasma membrane, facilitating interactions with membrane-bound substrates or effectors. DAG is represented in the membrane model (balls model) together with scaled C1A and B subdomains (blue spheres). It can be observed how the docking model proposed is compatible with the co-existence of hydrolysable and non-hydrolysable PtdIns(4,5)P\(_2\) molecules acting at the same time in the same PKCα molecule.
charges they contain, suggesting that the polybasic cluster forms a well-defined lipid-binding pocket that specifically fits the PtdIns(4,5)P\(_2\) molecule [9,17]. Thus the higher affinity of the C2 domain of cPKCs for PtdIns(4,5)P\(_2\), together with the high concentration of this lipid in the plasma membrane, probably facilitates membrane docking.

**PtdIns(4,5)P\(_2\) decreases the Ca\(^{2+}\) requirements of the C2 domains for membrane binding**

Additional studies have also demonstrated that the presence of PtdIns(4,5)P\(_2\) in the plasma membrane modulates the apparent Ca\(^{2+}\) affinity of these C2 domains to interact with membranes [13,14,17]. When the Ca\(^{2+}\)-dependence of C2 domains for membrane binding was studied, it was observed that the Ca\(^{2+}\) requirements were lower when PtdIns(4,5)P\(_2\) was present in POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)/POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine)-containing vesicles than when it was absent [17], especially in the case of PKC\(\alpha\) that exhibited the lowest Ca\(^{2+}\) needs, followed by PKC\(\gamma\) and \(\beta\) [17]. Most importantly, this effect was also correlated with the permanence of each domain in the plasma membrane of PC12 cells upon ATP stimulation [17].

**A plasma membrane docking model for cPKCs**

In conclusion, it has been demonstrated that the polybasic cluster in the C2 domain of cPKCs binds preferentially to PtdIns(4,5)P\(_2\) rather than to other phosphoinositides or other negatively charged phospholipids. Thus phosphatidylserine binds to the domain through the Ca\(^{2+}\)-binding region, with Ca\(^{2+}\) acting as a bridge between the protein and the membrane lipid, while PtdIns(4,5)P\(_2\) binds to the other distant motif, the polybasic cluster (Figure 2). All this evidence points to a new model for PKC\(\alpha\) activation, in which PtdIns(4,5)P\(_2\) itself would be a lipid target in the plasma membrane when Ca\(^{2+}\) spikes are generated in the cytosol. This raises an important and critical question about how to combine this new model with the classical activation model of this family of enzymes, in which PtdIns(4,5)P\(_2\) is hydrolysed to generate the DAG needed to activate PKC through its C1 domain. It has been demonstrated that several PtdIns(4,5)P\(_2\) pools exist in the plasma membrane, and so part of the phosphoinositide could be hydrolysed to generate DAG and another part would remain available to interact with the C2 domain of cPKCs. The application of newly developed tools to modulate PtdIns(4,5)P\(_2\) concentrations in intact cells, without generating second messengers such as DAG and Ins(1,4,5)P\(_3\), will be of great help to confirm this hypothesis [18,19].

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**References**


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