

Phosphatidylinositol 4,5-Bisphosphate Decreases the Concentration of Ca^{2+} , Phosphatidylserine and Diacylglycerol Required for Protein Kinase C α to Reach Maximum Activity

Antonio L. Egea-Jiménez, Ángel Pérez-Lara, Senena Corbalán-García, Juan C. Gómez-Fernández*

Departamento de Bioquímica y Biología Molecular-A, Facultad de Veterinaria, Regional Campus of International Excellence "Campus Mare Nostrum", Universidad de Murcia, Murcia, Spain

Abstract

The C2 domain of PKC α possesses two different binding sites, one for Ca^{2+} and phosphatidylserine and a second one that binds PIP $_2$ with very high affinity. The enzymatic activity of PKC α was studied by activating it with large unilamellar lipid vesicles, varying the concentration of Ca^{2+} and the contents of dioleoylglycerol (DOG), phosphatidylinositol 4,5-bisphosphate (PIP $_2$) and phosphatidylserine (POPS) in these model membranes. The results showed that PIP $_2$ increased the V_{max} of PKC α and, when the PIP $_2$ concentration was 5 mol% of the total lipid in the membrane, the addition of 2 mol% of DOG did not increase the activity. In addition PIP $_2$ decreases $K_{0.5}$ of Ca^{2+} more than 3-fold, that of DOG almost 5-fold and that of POPS by a half. The $K_{0.5}$ values of PIP $_2$ amounted to only 0.11 μM in the presence of DOG and 0.39 in its absence, which is within the expected physiological range for the inner monolayer of a mammalian plasma membrane. As a consequence, PKC α may be expected to operate near its maximum capacity even in the absence of a cell signal producing diacylglycerol. Nevertheless, we have shown that the presence of DOG may also help, since the $K_{0.5}$ for PIP $_2$ notably decreases in its presence. Taken together, these results underline the great importance of PIP $_2$ in the activation of PKC α and demonstrate that in its presence, the most important cell signal for triggering the activity of this enzyme is the increase in the concentration of cytoplasmic Ca^{2+} .

Citation: Egea-Jiménez AL, Pérez-Lara Á, Corbalán-García S, Gómez-Fernández JC (2013) Phosphatidylinositol 4,5-Bisphosphate Decreases the Concentration of Ca^{2+} , Phosphatidylserine and Diacylglycerol Required for Protein Kinase C α to Reach Maximum Activity. PLoS ONE 8(7): e69041. doi:10.1371/journal.pone.0069041

Editor: Mark S. Shapiro, University of Texas Health Science Center, United States of America

Received: March 22, 2013; **Accepted:** June 5, 2013; **Published:** July 10, 2013

Copyright: © 2013 Egea-Jiménez et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This paper has been supported by Grant No. BFU2011-22828 from Ministerio de Economía y Competitividad (Gobierno de España) and co-financed by FEDER (European Union) (<http://www.idi.mineco.gob.es/portal/site/MICINN/>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jcgomez@um.es

Introduction

PKC α (protein kinase C α) is a classical PKC isoenzyme that is activated by second messengers, namely the increase in Ca^{2+} concentration in the cytoplasm of the cell and the appearance of diacylglycerol in the membrane, where it establishes specific interactions with phosphatidylserine and PIP $_2$ [1].

The translocation of classical PKCs (cPKCs) to the plasma membrane is mediated by the C1 and C2 domains, and it has been shown that initial membrane affinity is mainly determined by C2 domain–membrane interactions, followed by C1 domain–diacylglycerol interactions [1]. One of the main sources of diacylglycerol in the plasma membrane following cell stimulation is PIP $_2$ which is hydrolyzed by phospholipase C to produce diacylglycerol and inositol 1,4,5-trisphosphate, which together activate protein kinase C for sustained cellular responses [2]. However, it has been shown that PIP $_2$ may also activate PKC α by direct binding to a polylysine motif located in strands $\beta 3$ and $\beta 4$ [3–7] and that can be considered a specific site for PIP $_2$ [8] (see Fig. 1). Other molecules like phosphatidylserine or phosphatidic acid [9] or even retinoic acid [10] may also bind with lower affinity to this site. It has been clearly shown that PIP $_2$ is important for PKC α translocation to the

membrane and for prolonging this translocation. Rapid [5,11,12] kinetics studies on the binding of this enzyme to model membranes suggested that the interaction of PKC α with membranes occurs via two steps: a rapid weak recruitment to the membrane due to non-specific interactions with (primarily) anionic lipids and the formation of a high affinity complex due to stereospecific interactions of each PKC α domain with its specific ligands [12].

PKC α enzyme is a paradigmatic example for bearing a C2 domain which may simultaneously bind three different activators, in this case Ca^{2+} , phosphatidylserine and PIP $_2$. Fig. 1 shows this C2 domain in which Ca^{2+} binds to its site, acting as a bridge for phosphatidylserine, although this phospholipid also directly interacts with several protein residues [13,14]. In another site located in a β -groove, PIP $_2$ binds with great affinity.

Previous work has shown that PKC α exhibits high cooperativity in its activity by phosphatidylserine [15,16] and that the two second messengers of the kinase, diacylglycerol and Ca^{2+} , markedly increase the affinity of the kinase for phosphatidylserine [17]. In this paper, we use highly purified full-length PKC α to perform a kinetic study of the activation of PKC α by model membranes, in which the concentrations of POPS, DOG, PIP $_2$

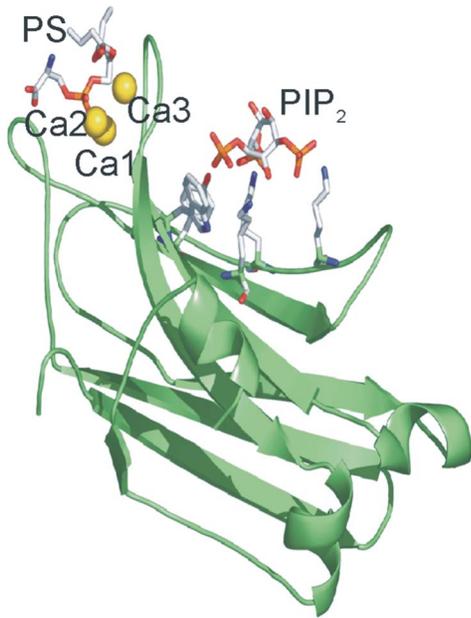


Figure 1. Structure of PKC α C2 domain bound to Ca²⁺-POPS-PIP₂ in a quaternary complex. The C2 molecule is shown in green. The three calcium ions are shown in yellow spheres, one of them bridging the protein with phosphatidylserine (PS) at the tip of the domain. The PIP₂ molecule is bound to the β 3- β 4 chains [8]. PDB accession number 3GPE.
doi:10.1371/journal.pone.0069041.g001

and Ca²⁺ are varied. Our results indicate that PIP₂ enhances PKC α activity and decreases the required concentrations of the other activators, to reach maximum activities.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), L- α -phosphatidylinositol 4,5-bisphosphate (PIP₂) and 1,2-*sn*-dioleoylglycerol (DOG) were purchased from Avanti Polar Lipids (Alabaster, ALA, U.S.A.). To discard any 1,2-diacyl-*sn*-glycerol contamination of the PIP₂ source used, L- α -phosphatidylinositol 4,5-bisphosphate (PIP₂) (lot BPIP2 (4,5)-54) was analyzed by Avanti Polar Lipids and their release testing included Gravimetric analysis, Phosphorus analysis, Proton and Phosphorus NMR, Mass Spectrometry, Thin Layer Chromatography (TLC), HPLC and Calcium by ICP/MS without detecting any impurity. The HPLC assay indicated 100% PIP₂. In addition, once dissolved it was also analysed in our laboratory by using TLC and no impurity was detected either. All other chemicals were of high purity and supplied by Sigma Chemical Co. (Madrid, Spain).

Expression and Purification of Protein Kinase C α

The full length cDNA for rat PKC α was kindly provided by Profs. Ono and Nishizuka (Kobe, Japan). PKC α was cloned into the plasmid pFastBac HT (Invitrogen, Madrid, Spain). A 0.5 litre scale culture of Sf9 insect cells (*Spodoptera frugiperda*) at 2.1 \times 10⁶ cells/ml was infected with the recombinant baculovirus. Cells were harvested 60 h postinfection (cell viability 80%), pelleted at 4500 rpm for 20 min, and resuspended in buffer containing 25 mM Tris-HCl pH 7.5, 100 μ M EGTA, 50 mM NaF, 100 μ M NaVO₃, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM

PMSF, 10 μ g/ml leupeptin and 10 mM benzamide. The pellet was disrupted by sonication (6 \times 10 s) and the resulting lysate was centrifuged at 15000 rpm for 20 min. The supernatant was applied to a 1 ml His-Gravi TrapTM column (GE Healthcare, Barcelona, Spain) and equilibrated with 25 mM Tris-HCl pH 7.5, 150 mM NaCl and 20 mM imidazole buffer. The bound proteins were eluted by an imidazole gradient (20–500 mM). Fractions containing protein kinase C α from a His-Gravi TrapTM column were pooled, concentrated by ultrafiltration to a 2 mL volume and adjusted by the addition of 5 M NaCl to give a NaCl concentration of 1 M.

This fraction was then processed by hydrophobic exchange chromatography, directly applying it to a SOURCE 15PHE 4.6/100 PE[®] column[®] Tris-HCl pH 7.5, 1 mM DTT and 10% glycerol. After the unbound proteins had passed through the column, PKC was eluted with a gradient of 0.8–0 M NaCl. Highly pure PKC α was obtained, as determined by SDS-PAGE (12.5%). The protein was aliquoted and stored at -80° C in the presence of 10% (w/v) glycerol and 0.05% (v/v) Triton X-100.

Preparation of Phospholipid Vesicles

Lipid vesicles were generated by mixing chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) and L- α -phosphatidylinositol-4,5-bisphosphate (PIP₂) in the desired proportions. Lipids were dried from the organic solvent under a stream of oxygen-free nitrogen, and then the last traces of organic solvent were removed under vacuum for at least 4 h. Dried phospholipids were resuspended in the corresponding buffers by vigorous vortexing and then large unilamellar phospholipid vesicles of about 100 nm diameter were prepared by extruding (11 times) rehydrated phospholipid suspensions through two stacked 0.1 nm polycarbonate membranes (Millipore Inc., Bedford, MA, USA).

Enzymatic Activity Assay

Enzymatic activity was assayed using a technique described previously [17], in which the incorporation of radioactive phosphate [γ -³²P] to kinase substrate (histone III-S) was measured. Lipids in organic solutions were mixed in the desired proportions and dried under a stream of N₂, removing the last traces of organic solvent by keeping the samples under vacuum for at least four hours. Large unilamellar vesicles (LUVs) were prepared using the extrusion technique, as explained above. These lipids were resuspended immediately before use in a buffer composed of 20 mM Tris-HCl (pH 7.5), 0.5 mg/ml of histone III-S, 40 μ M ATP [γ -³²P] (3000,000 cpm/nmol), 5 mM MgCl₂ 1 mM EGTA and enough CaCl₂ to give a free Ca²⁺ concentration of 200 μ M, except in the assays in which this concentration was varied. The necessary concentrations of CaCl₂ added in each case were calculated by using the procedure described in [18]. The final concentration of lipids in the reaction mixture was 625 μ M. The reaction was started by the addition of 5 μ l of PKC α (0.004 μ g/ml). After 30 min at 25 $^{\circ}$ C, the reaction was stopped with 1 ml of ice-cold 25% (w/v) trichloroacetic acid (TCA) and 1 ml of ice-cold 0.05% (w/v) bovine serum albumin. After precipitation on ice for 30 min, the protein precipitate was collected on a 2.5 cm glass filter (Sartorius, Göttingen, Germany) and washed with 10 ml of ice-cold 10% trichloroacetic acid. The amount of ³²Pi incorporated in histone was measured by liquid scintillation counting. The linearity of the assay was confirmed from the time-course of histone phosphorylation over a 30 min period. Additional control experiments were run in the absence of calcium to measure basal

kinase activity only adding EGTA without any CaCl_2 with a reaction time of 30 minutes.

Data Analysis

The dependence of PKCα activity on the contents of the different activators in the model membranes was analyzed by a non-linear least squares fit to a modified Hill equation:

$$y = a + V_{\max} \left[\frac{x^n}{K_{0.5} + x^n} \right]$$

where y is the measured activity of PKCα, a is the activity in the absence of lipid or Ca^{2+} (background), V_{\max} is the lipid-stimulated activity, x is the concentration of the activator, $K_{0.5}$ is the concentration of activator resulting in half maximal activity and n is the Hill coefficient. Standard errors for n , V_{\max} and $K_{0.5}$, taken for three independent experiments, are reported.

Results

The important contribution of PIP₂ to PKCα enzymatic activity was clearly observed when it was studied as a function of Ca^{2+} concentration. A POPC/POPS molar ratio of about 4 was used in these assays since the concentration of POPS in the inner monolayer of eukaryotic plasma membranes, such as in erythrocyte or platelet cells, is roughly this [19–21]. The physiological concentration of PIP₂ has been described to be around 1 mol% of the total lipid of plasma membranes [22,23] and it is likely to be concentrated in the inner monolayer at 2 mol%, which increase locally if it forms clusters or patches [24]. As regards diacylglycerol, the physiological levels of this lipid in biomembranes were reviewed in [25]. For example, quantitative measurements of diacylglycerols present in stimulated cells have shown that they may reach 1.45 mol% of the total lipid concentration [26] or about 2 mol% [27]. So the concentrations of diacylglycerol used in this work can be considered physiological and well within the range of diacylglycerol concentrations used in standard procedures for PKC activation assays, which use values similar to those used here [28] or even as high as 11.5 mol% with respect to total lipid [29] or as 19 mol% [30] or 25 mol% [31]. In enzymatic studies where the effect of lipid concentrations were studied, 200 μM Ca^{2+} was used in order to ensure that this cation was not a limiting factor.

When the dependence of PKCα activity on Ca^{2+} concentration was studied (Fig. 2) the sigmoidal curves obtained in all cases, pointed to cooperativity. In the presence of POPC/POPS (80:20 molar ratio) alone, increasing concentrations of Ca^{2+} led to a cooperative increase in activity, with a $K_{0.5}$ of 1.30 μM in Ca^{2+} (see Table 1), rising from 107.6 nmol Pi/min/mg at 0.1 μM in Ca^{2+} to a V_{\max} of 898.4 nmol Pi/min/mg and a Hill coefficient of 2.28. If DOG was added to the membrane to give a composition of POPC/POPS/DOG (78:20:2 molar ratio) the cooperative behavior was again present, but now the $K_{0.5}$ was 0.84 μM in Ca^{2+} , with the activity raising from 70.5 nmol Pi/min/mg at 0.1 μM Ca^{2+} to a V_{\max} of 1192.7 nmol Pi/min/mg and a Hill coefficient of 2.42. It is clear that in the presence of DOG, the $K_{0.5}$ for Ca^{2+} decreases, and there is an increase in V_{\max} .

When PIP₂ was added to the membrane to give a composition of POPC/POPS/PIP₂ (75:20:5 molar ratio), $K_{0.5}$ was now 0.59 μM of Ca^{2+} (Table 1), the activity was 597.4 nmol Pi/min/mg at 0.1 μM of Ca^{2+} and V_{\max} was 1790.7 nmol Pi/min/mg at 10 μM of Ca^{2+} , the higher cooperativity giving a Hill coefficient of 4.10. Even higher cooperativity (Hill coefficient of 8.61) was observed for a membrane which incorporated also DOG, namely

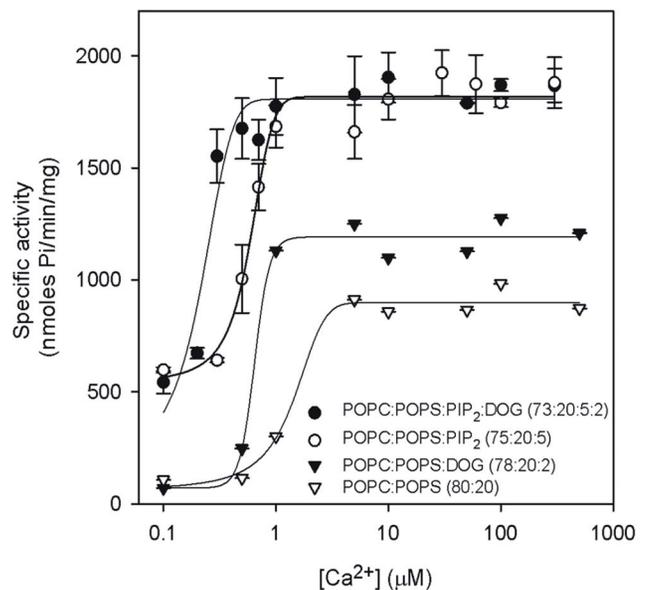


Figure 2. The dependence of PKCα activity on Ca^{2+} concentration. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 μmol. SD calculated from 3 independent experiments. doi:10.1371/journal.pone.0069041.g002

PC/PS/PIP₂/DOG (73:20:5:2 molar ratio), although the activity levels did not change with respect to the membrane without DOG, being now 512.9 nmol Pi/min/mg at 0.1 μM of Ca^{2+} while V_{\max} was 1804.9 nmol Pi/min/mg at 10 μM of Ca^{2+} . It is interesting that at very low concentration of Ca^{2+} , e.g. 0.1 μM, the activity in the presence of PIP₂ was higher than in the absence of this phosphoinositide, both in the presence and in the absence of DOG. However, the addition of 2% DOG did not increase the activity levels when 5 mol% PIP₂ was present.

The effect of increasing POPS concentration was also studied (Fig. 3). When PIP₂ was absent in a POPC/POPS/DOG mixture (98-x:x:2 molar ratio), the effect observed was of positive cooperativity (very high Hill coefficient of 13.18), with a $K_{0.5}$ = 15.12 mol% of POPS (Table 2), which is similar to the effect observed previously for membrane activation of this enzyme [14]. V_{\max} was 1260.2 nmol Pi/min/mg.

If PIP₂ was also present, at just 1 mol%, in a mixture containing POPC/POPS/PIP₂/DOG (97-x:x:1:2 molar ratio), positive cooperativity was still observed, with a Hill coefficient of 3.71, an initial activity of 490 nmol Pi/min/mg in the absence of POPS and a V_{\max} of 1586.2 nmol Pi/min/mg. $K_{0.5}$ was 15.4 mol% of POPS.

Table 1. Dependence of PKCα activation on Ca^{2+} .

Lipid mixture	$K_{0.5}$	V_{\max} (nmol Pi/min/mg)	n
POPC:POPS (80:20)	1.30±0.08	898.2±27.8	2.28±0.89
POPC:POPS:DOG (78:20:2)	0.84±0.27	1192.7±96.3	2.42±0.69
POPC:POPS:PIP ₂ (75:20:5)	0.58±0.09	1790.7±64.4	4.10±0.92
POPC:POPS:PIP ₂ :DOG (73:20:5:2)	0.26±0.01	1804.9±104.4	8.61±0.27

$K_{0.5}$, n and V_{\max} were obtained by nonlinear least square fit of the data in the equation described in the Methods Section. doi:10.1371/journal.pone.0069041.t001

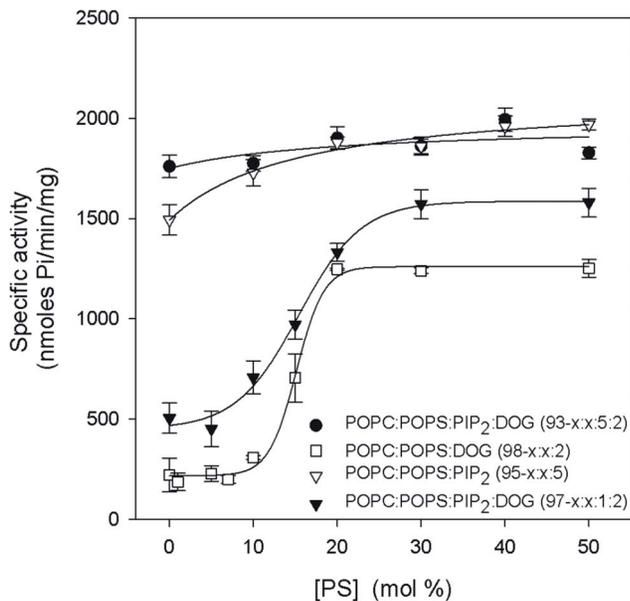


Figure 3. The dependence of PKCa activity on the POPS molar percentage in the vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 μmol . SD calculated from 3 independent experiments.

doi:10.1371/journal.pone.0069041.g003

If PIP₂ was present in the lipid mixture, but with no DOG, POPC/POPS/PIP₂ (95-x:x:5), the initial activity, even in the absence of POPS, was already high, with a value of 1492 nmol Pi/min/mg. V_{max} reached a value of 2084.1 nmol Pi/min/mg and $K_{0.5}$ was 13.94 mol% of POPS. Thus the addition of PIP₂ decreased $K_{0.5}$ even if DOG was not present, and the activity was almost saturated and no apparent cooperativity was observed ($n = 1.10$).

Fig. 3 also shows that when PIP₂ was increased to 5 mol%, to give a lipid mixture of POPC/POPS/PIP₂/DOG (93-x:x:5:2 molar ratio), a very small increase in activity was already observed when POPS was increased since nearly maximum activity was observed in the absence of POPS (1760 nmol Pi/min/mg) and V_{max} was 1895.8 nmol Pi/min/mg, with $K_{0.5}$ of 8.20 mol% POPS and a Hill coefficient of 1.50.

Fig. 4 shows the activity studied as function of DOG concentration. When the membrane was composed of POPC/POPS/DOG (75-x:25:x), $K_{0.5}$ was 0.82 mol% of DOG (Table 3). The activity was 666 nmol Pi/min/mg at 0 mol% of DOG and rose to give a V_{max} of 1307.9 nmol Pi/min/mg and a Hill coefficient of 1.59, indicating low positive cooperativity. When PIP₂ was incorporated into this assay at just 1 mol% in a POPC/

POPS/PIP₂/DOG (74-x:25:1:x molar ratio) mixture, $K_{0.5}$ was 0.38 mol% of DOG, which was notably lower than the 1.10 mol% observed in the absence of PIP₂, while the Hill coefficient showed little change (1.39). When the PIP₂ concentration was raised to 5 mol%, to give a POPC/POPS/PIP₂/DOG mixture (70-x:25:5:x molar ratio), $K_{0.5}$ decreased to 0.17 mol% DOG, although V_{max} maintained a similar value of 1701.9 nmol Pi/min/mg and the Hill coefficient (0.47) indicated an apparent negative cooperativity.

In another set of experiments, the concentration of PIP₂ was varied in the presence and in the absence of DOG, keeping the Ca^{2+} concentration constant at 200 μM . Fig. 5 depicts the results obtained when the molar percentage in the membrane of PIP₂ was increased in the absence of DOG, POPC/POPS/DOG (75-x:25:x). As can be seen, the $K_{0.5}$ was 0.39 (Table 4) and the V_{max} 1816.2 nmol Pi/min/mg, with low positive cooperativity ($n = 1.60$). In the presence of 2 mol% with a POPC/POPS/PIP₂/DOG membrane (73-x:25:x:2, molar ratio) $K_{0.5}$ decreased to 0.11 mol% PIP₂. This is interesting since it clearly demonstrates that very low concentrations (well below physiological concentrations) are sufficient to significantly enhance the activity of PKCa.

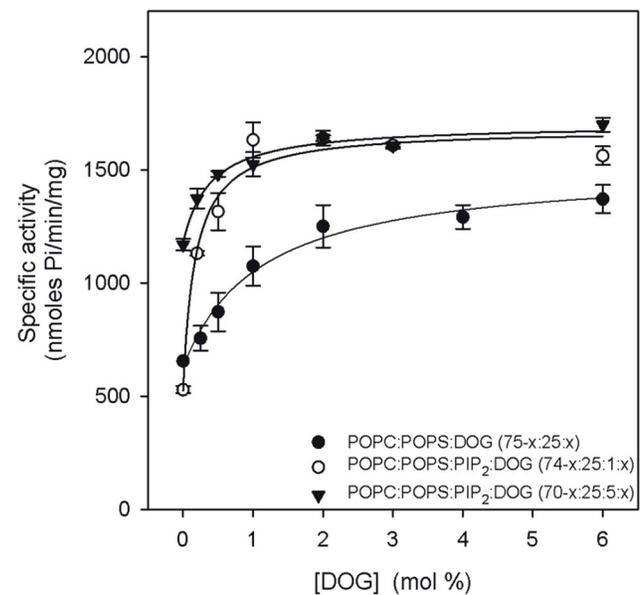


Figure 4. The dependence of PKCa activity on the DOG molar percentage in the lipid vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca^{2+} concentration was 200 μmol . SD calculated from 3 independent experiments.

doi:10.1371/journal.pone.0069041.g004

Table 2. Dependence of PKCa activation on POPS.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	n
POPC:POPS:DOG (98-x:x:2)	15.12 ± 0.24	1260.2 ± 48.4	13.18 ± 1.9
POPC:POPS:PIP ₂ :DOG (97-x:x:1:2)	15.40 ± 0.71	1586.2 ± 71.1	3.71 ± 0.77
POPC:POPS:PIP ₂ (95-x:x:5)	13.94 ± 1.45	2084.0 ± 73.6	1.10 ± 0.39
POPC:POPS:PIP ₂ :DOG (93-x:x:5:2)	8.20 ± 0.84	1895.8 ± 101.3	1.50 ± 0.56

$K_{0.5}$, n and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Methods Section.

doi:10.1371/journal.pone.0069041.t002

Table 3. Dependence of PKC α activation on DOG.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	n
POPC:POPS:DOG (75-x:25:x)	0.82 \pm 0.08	1307.9 \pm 52.7	1.59 \pm 0.22
POPC:POPS:PIP ₂ :DOG (74-x:25:1:x)	0.38 \pm 0.10	1681.7 \pm 85.9	1.39 \pm 0.01
POPC:POPS:PIP ₂ :DOG (70-x:25:5:x)	0.17 \pm 0.05	1701.9 \pm 69.2	0.47 \pm 0.15

$K_{0.5}$, n and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Methods Section.
doi:10.1371/journal.pone.0069041.t003

V_{max} was 1857.6 nmol Pi/min/mg in this case, which is not significantly different with respect to the mixture without DOG, but the Hill coefficient was now 0.78, indicating apparent negative cooperativity during PIP₂ activation.

Discussion

This study was designed to show the importance of PIP₂ for the catalytic activity of PKC α . Previous studies have shown that PIP₂ binds to a site located in the β 3- and β 4-sheets of the C2 domain, which is a conserved site found also in other C2 domains (Fig. 1) [8]. It was also shown that the presence of PIP₂ considerably increased the kinase activity of PKC α [3]. More recently the binding of PKC α to model membranes was studied by monitoring rapid kinetics [12]. It was observed that, in the absence of PIP₂, the protein rapid rate of binding was especially dependent on the POPS concentration and a high affinity complex evolved more slowly, which implies specific interactions with POPS and DOG. Both association and dissociation constants were decreased by the presence of PIP₂, but the association equilibrium constant was increased, i.e. the species of PKC α bound to the membrane was favored. A model was proposed in which PKC α binds to the membranes via a two-step mechanism consisting of a rapid initial

recruitment of PKC α to the membrane driven by interactions with POPS and/or PIP₂, although interactions with DOG are also involved. PKC α carries out a 2-D search on the lipid bilayer to establish specific interactions with its specific ligands. In this way the longer time of residence of the enzyme in the bound state induced by PIP₂ could explain its activating effect.

In this work we have assayed in detail the enzymatic activity of PKC α changing the concentrations of the different activators in order to obtain a comprehensive picture of the way in which PIP₂ may affect the catalytic action of this enzyme.

With respect to the dependence on Ca²⁺, it was observed that, as it has long been known [32], the addition of DOG to POPC/POPS increased to activity, the V_{max} going from 898.2 to 1192.7 nmol Pi/min.mg (Table 1), and decreased $K_{0.5}$ [33], but the Hill coefficient was not significantly altered, showing positive cooperativity. Therefore, the binding of DOG to the C1 domain will influence Ca²⁺ binding to the C2 domain, facilitating the binding of the C2 domain to the membrane at lower Ca²⁺ concentrations, an increase in activity that can only be due to its capacity to retain the C1 domain bound to the membrane, impeding return to the inactive structure of PKC.

The addition of PIP₂ in the absence of DOG increased V_{max} with respect to a membrane containing POPC/POPS/DOG (1790.7 compared to 1192.7 nmol Pi/min.mg) and the further addition of DOG led to the maximum activation (V_{max} reaching 1804.9 nmol Pi/min.mg). However, the effect of DOG in this case was not great since the activity was already close to the maximum in the absence of DOG. Note that when PIP₂ is introduced and POPC/POPS/DOG and POPC/POPS/DOG/PIP₂ are compared, the $K_{0.5}$ for Ca²⁺ is reduced in the presence of PIP₂ more than three-fold (from 0.84 to 0.26 μ M) and the Hill coefficient increases from 2.42 to 8.61. Since it is known that the presence of PIP₂ collaborates in the anchoring of the C2 domain to the membrane [3,8,12,34] and that Ca²⁺ is necessary to allow the binding of the protein to the membrane, it is clear that there is an interplay between these activators. It seems that the presence of PIP₂ lowers the amount of Ca²⁺ required for binding and activity and, at the same time, increases the cooperativity for the binding of Ca²⁺. Indeed, it has been shown that PIP₂ markedly reduces the

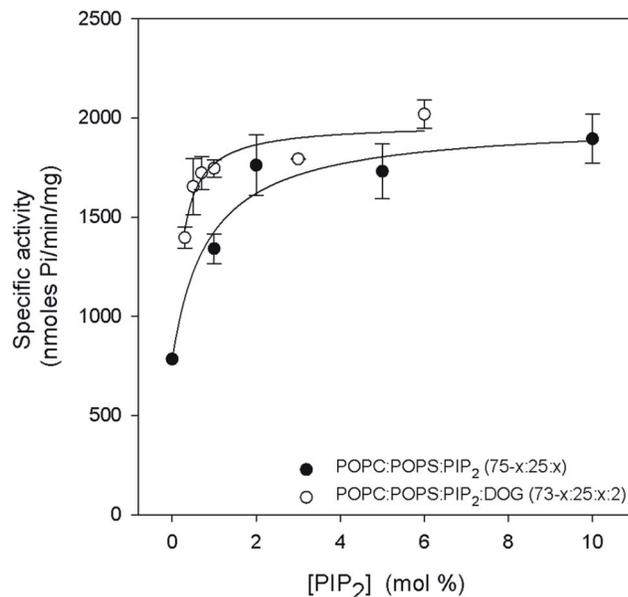


Figure 5. The dependence of PKC α activity on the PIP₂ molar percentage in the lipid vesicles. The molar ratios of the lipid components of the vesicles used to activate the enzyme are shown. Ca²⁺ concentration was 200 μ mol. SD calculated from 3 independent experiments.
doi:10.1371/journal.pone.0069041.g005

Table 4. Dependence of PKC α activation on PIP₂.

Lipid mixture	$K_{0.5}$	V_{max} (nmol Pi/min/mg)	n
POPC:POPS:PIP ₂ (75-x:25:x)	0.39 \pm 0.61	1816.2 \pm 143.5	1.60 \pm 0.03
POPC:POPS:PIP ₂ :DOG (73-x:25:x:2)	0.11 \pm 0.02	1857.6 \pm 73.9	0.78 \pm 0.15

$K_{0.5}$, n and V_{max} were obtained by nonlinear least square fit of the data in the equation described in the Methods Section.
doi:10.1371/journal.pone.0069041.t004

concentration of Ca²⁺ required for the binding of isolated C2 domain [4,34]. It is known that up to 3 Ca²⁺ ions may bind at the calcium binding site of the C2 domain of PKC α [9,35]. A sequential model for classical PKC membrane binding and activation has been proposed [3,5,36], whereby the increase in intracellular Ca²⁺ produces the binding of Ca1 and Ca2 when the protein is still in the cytosol, leading to the membrane being targeted by the enzyme through the C2 domain. Ca1 is responsible for bridging the protein to the phospholipid molecules (Fig. 1), which are also recognized with the help of Asn189 and Arg216, whereas Ca2 is responsible for keeping Ca1 in its proper location and for inducing a conformational change in PKC, which partially penetrates and docks in the phospholipid bilayer by means of CBR3 (Arg249 and Thr251). Once recruited to the membrane, a third Ca²⁺ binds, stabilizing the C2 domain-membrane complex. This enables PKC to reside in the membrane for a longer time, allowing the C1 domain to find the diacylglycerol generated upon receptor stimulation [14,33,37–43].

In the case of PKC α dependence on POPS, V_{max} was clearly increased by the addition of 1 mol% of PIP₂, and the addition of 5 mol% in the absence of DOG led to very high activities, which did not increase even when DOG was added. This is an interesting result and confirms the great activation capacity of PIP₂, and shows that fixing the C2 domain of PKC α to the membrane through the calcium binding site and the PIP₂ site decreases the importance of the C1 domain respect to activity. However, the addition of DOG to the membrane containing 5 mol% PIP₂ reduced $K_{0.5}$ from 13.94 to 8.20 μ M, showing that binding of the C1 domain may also play a role.

It has been described that POPS binds cooperatively to PKC, with a stoichiometry of 4 [44], ≥ 12 [15] or approximately 8 [45] lipid molecules per molecule of protein. A number of authors have observed apparent cooperativity for the activation in mixed micelles with Triton X-100, leading to high Hill coefficients (higher than 8 [46–48] or about 5 [49]) but when the activation was carried out with lipid vesicles, the Hill coefficients were about 2.6 [50] or close to 1 [49]. In our case, a high degree of positive cooperativity was observed in the absence of PIP₂, with a Hill coefficient of 13.18. The use of different types of vesicles in the studies mentioned above may be the reason for the disparity of the results. However, the addition of just 1 mol% of PIP₂ reduced n to 3.71, and at 5 mol% PIP₂ no cooperativity was evident. Just one POPS molecule is known to bind to the C2 domain of PKC α [8], although more POPS molecules may bind to the C1 domain [51]. It is interesting in this respect that Hill coefficients close to 1 were observed for the binding of the isolated C2 domain to phospholipid vesicles (A. Torrecillas, Ph.D. Thesis, University of Murcia, 2003).

However, it is nowadays recognized that a number of mechanisms may lead to kinetic cooperativity in the absence of true cooperative interactions, and kinetic models have been suggested to explain the apparent cooperativity observed for the binding of lipid to PKC, for example, proposing ligand trapping [52] or effects specific to the interaction with multiple membrane associated ligands [45] have been suggested, the last authors observing that the apparent cooperativity may be abolished in conditions where membrane binding involves a non-phosphatidylserine mechanism, as in the presence of activators such as phorbol esters. This explains why PIP₂ reduces the apparent cooperativity so drastically. Therefore, Hill indexes obtained for the binding of proteins, such as PKC to lipids in vesicles or in micelles, may be informative as regards threshold-binding and how this type of binding may be regulated by different ligands.

Diacylglycerol also plays a role, especially in the absence of PIP₂, but in the presence of the phosphoinositide its role is reduced. The reason for that may be related to the tighter anchoring of the enzyme as seen by stopped flow experiments using the full-length enzyme [12] and the different orientation of the C2 domain with respect to the membrane, as seen by studying the membrane docking of this domain [53]. These effects occasioned by the interaction with PIP₂ may prolong the activated state. The interplay between DOG and PIP₂ was also evident when DOG was changed. Even at 1 mol% of PIP₂, $K_{0.5}$ decreased from 1.10 mol% of DOG in its absence to 0.38 in its presence. If PIP₂ was 5 mol%, then $K_{0.5}$ further decreased to 0.17 mol% of DOG, a substantial decrease compared with the total absence of the phosphoinositide. This illustrates that in the presence of PIP₂ the enzyme is tightly bound to the membrane and small concentrations of DOG are sufficient to maintain the activity. It is interesting that small apparent positive cooperativity was detected in the absence of PIP₂ ($n = 1.59$), which was reduced following the addition of 1 mol% PIP₂. This apparent cooperativity in the binding of DOG may not necessarily reflect that the two C1 subdomains bind to DOG when its concentration is sufficiently high, but may be explained by the apparent cooperativity effect described above for POPS, while PIP₂ will reduce the apparent cooperativity due to its increasing of the membrane affinity of the protein. It is remarkable, that at 5 mol% of PIP₂ the Hill coefficient (0.47) indicated apparent negative cooperativity for diacylglycerol, which might be explained by a change in membrane structure at relatively high DOG concentrations [25].

It is interesting that very low $K_{0.5}$ values were observed for PIP₂ even in the absence of DOG, the value (0.39 μ M) being within the physiological range of concentrations. In the presence of DOG a very reduced $K_{0.5}$ value of 0.11 μ M was observed for PIP₂, although V_{max} increased very slightly as a result of the addition of DOG, confirming that in the presence of PIP₂ diacylglycerol is playing a relatively secondary role in the activation of PKC α . Low K_D values have been reported for the binding of PIP₂ to the isolated C2 domain of PKC α [4,54] with about 1.9 μ M for POPC-POPS-PIP₂ vesicles, a value which is compatible with our observations for $K_{0.5}$.

Taken together, the results show that PIP₂ increases the V_{max} of PKC α and that when its concentration is 5 mol%, the addition of 2 mol% of DOG does not further increase the activity. Moreover, this concentration decreases $K_{0.5}$ for Ca²⁺ more than 3-fold, almost 5-fold that of DOG and by a half that of POPS. It is also noteworthy that $K_{0.5}$ values for PIP₂ amounted to only 0.11 μ M in the presence of DOG and 0.39 in its absence, therefore well below the maximum physiological concentration for the inner monolayer of a mammalian plasma membrane. As a consequence, PKC α may be expected to operate near its maximum capacity even in the absence of a cell signal producing diacylglycerol. Nevertheless, we have shown that the presence of DOG may also help, since $K_{0.5}$ for PIP₂ notably fell in its presence. On the other hand, since Ca²⁺ has been shown to be essential for the binding of PIP₂ to the C2 domain of PKC α [4,54], this enzyme may be triggered simply by an increase in the cytoplasm concentration of this cation. Since it has been shown that the other classical isoenzymes of PKC are similar to PKC α as regards to the affinity of their C2 domains for PIP₂ [4], the above observations may well be extended to them.

In conclusion, the results obtained in this work are compatible with the sequential mechanism previously proposed (3) and further confirmed in vivo (5). Basically, intracytosolic Ca²⁺ elevations are the trigger to translocate PKC α to the plasma membrane. Once there, two situations can be found: in microdomains enriched only

with phosphatidylserine, the docking of the C2 domain is not enough to liberate the catalytic domain for substrate access, and as seen in the 3D structure recently solved [55], the C1B domain might still keep blocking the catalytic domain. Due to this, the presence of 1,2-diacyl-*sn*-glycerol in the lipid vesicles by docking at least the C1A domain enables the enzyme to gain its full activation [56]. A second situation can be found when the microdomains are enriched in phosphatidylserine and PIP₂ at the plasma membrane. In this case, the C2 domain docks in a different orientation since it has to anchor through two different points, i.e. the CBR (Ca²⁺/PS) and the lysine rich cluster (PIP₂), this might induce a conformational change that unleash the C1 domain from the blocking conformation and enables the catalytic domain to access the

substrate and consequently full activation of the enzyme. Whether the C1 domains can interact with the membrane independently of 1,2-diacyl-*sn*-glycerol is not known but there are previous reports indicating that the C1 domains can interact unspecifically with negatively charged phospholipids through the Arg and Lys residues located in its surface [57].

Author Contributions

Conceived and designed the experiments: JCGF SCG. Performed the experiments: ALEJ APL. Analyzed the data: ALEJ APL SCG JCGF. Contributed reagents/materials/analysis tools: JCGF SCG ALEJ APL. Wrote the paper: JCGF. Enzyme assays: ALEJ APL.

References

- Corbalan-Garcia S, Gomez-Fernandez JC (2006) Protein kinase C regulatory domains: the art of decoding many different signals in membranes. *Biochim Biophys Acta* 1761: 633–654.
- Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9: 484–496.
- Corbalan-Garcia S, Garcia-Garcia J, Rodriguez-Alfaro JA, Gomez-Fernandez JC (2003) A new phosphatidylinositol 4,5-bisphosphate-binding site located in the C2 domain of protein kinase Calpha. *J Biol Chem* 278: 4972–4980.
- Guerrero-Valero M, Marin-Vicente C, Gomez-Fernandez JC, Corbalan-Garcia S (2007) The C2 domains of classical PKCs are specific PtdIns(4,5)P₂-sensing domains with different affinities for membrane binding. *J Mol Biol* 371: 608–621.
- Marin-Vicente C, Nicolas FE, Gomez-Fernandez JC, Corbalan-Garcia S (2008) The PtdIns(4,5)P₂ ligand itself influences the localization of PKCalpha in the plasma membrane of intact living cells. *J Mol Biol* 377: 1038–1052.
- Sanchez-Bautista S, Marin-Vicente C, Gomez-Fernandez JC, Corbalan-Garcia S (2006) The C2 domain of PKC alpha is a Ca²⁺-dependent PtdIns(4,5)P₂ sensing domain: A new insight into an old pathway. *Journal of Molecular Biology* 362: 901–914.
- Marin-Vicente C, Gomez-Fernandez JC, Corbalan-Garcia S (2005) The ATP-dependent membrane localization of protein kinase Calpha is regulated by Ca²⁺ influx and phosphatidylinositol 4,5-bisphosphate in differentiated PC12 cells. *Mol Biol Cell* 16: 2848–2861.
- Guerrero-Valero M, Ferrer-Orta C, Querol-Audi J, Marin-Vicente C, Fita I, et al. (2009) Structural and mechanistic insights into the association of PKCalpha-C2 domain to PtdIns(4,5)P₂. *Proc Natl Acad Sci U S A* 106: 6603–6607.
- Ochoa WF, Corbalan-Garcia S, Eritja R, Rodriguez-Alfaro JA, Gomez-Fernandez JC, et al. (2002) Additional binding sites for anionic phospholipids and calcium ions in the crystal structures of complexes of the C2 domain of protein kinase C alpha. *Journal of Molecular Biology* 320: 277–291.
- Ochoa WF, Torrecillas A, Fita I, Verdaguer N, Corbalan-Garcia S, et al. (2003) Retinoic acid binds to the C2-domain of protein kinase C(alpha). *Biochemistry* 42: 8774–8779.
- Evans JH, Murray D, Leslie CC, Falke JJ (2006) Specific translocation of protein kinase Calpha to the plasma membrane requires both Ca²⁺ and PIP₂ recognition by its C2 domain. *Mol Biol Cell* 17: 56–66.
- Perez-Lara A, Egea-Jimenez AL, Ausili A, Corbalan-Garcia S, Gomez-Fernandez JC (2012) The membrane binding kinetics of full-length PKCalpha is determined by membrane lipid composition. *Biochim Biophys Acta* 1821: 1434–1442.
- Verdaguer N, Corbalan-Garcia S, Ochoa WF, Fita I, Gomez-Fernandez JC (1999) Ca²⁺ bridges the C2 membrane-binding domain of protein kinase Calpha directly to phosphatidylserine. *EMBO J* 18: 6329–6338.
- Conesa-Zamora P, Lopez-Andreo MJ, Gomez-Fernandez JC, Corbalan-Garcia S (2001) Identification of the phosphatidylserine binding site in the C2 domain that is important for PKC alpha activation and in vivo cell localization. *Biochemistry* 40: 13898–13905.
- Newton AC, Koshland DE Jr (1989) High cooperativity, specificity, and multiplicity in the protein kinase C-lipid interaction. *J Biol Chem* 264: 14909–14915.
- Hamun YA, Loomis CR, Bell RM (1985) Activation of Protein Kinase-C by Triton X-100 Mixed Micelles Containing Diacylglycerol and Phosphatidylserine. *Journal of Biological Chemistry* 260: 39–43.
- Orr JW, Newton AC (1992) Interaction of protein kinase C with phosphatidylserine. 2. Specificity and regulation. *Biochemistry* 31: 4667–4673.
- Chang D, Hsieh PS, Dawson DC (1988) Calcium: a program in BASIC for calculating the composition of solutions with specified free concentrations of calcium, magnesium and other divalent cations. *Comput Biol Med* 18: 351–366.
- Verkleij AJ, Zwaal RF, Roelofs B, Comfurius P, Kastelijn D, et al. (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim Biophys Acta* 323: 178–193.
- Chap HJ, Zwaal RF, van Deenen LL (1977) Action of highly purified phospholipases on blood platelets. Evidence for an asymmetric distribution of phospholipids in the surface membrane. *Biochim Biophys Acta* 467: 146–164.
- Leventis PA, Grinstein S (2010) The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys* 39: 407–427.
- McLaughlin S, Wang J, Gambhir A, Murray D (2002) PIP₂ and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct* 31: 151–175.
- Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443: 651–657.
- Huang S, Lifshitz L, Patki-Kamath V, Tuft R, Fogarty K, et al. (2004) Phosphatidylinositol-4,5-bisphosphate-rich plasma membrane patches organize active zones of endocytosis and ruffling in cultured adipocytes. *Mol Cell Biol* 24: 9102–9123.
- Gomez-Fernandez JC, Corbalan-Garcia S (2007) Diacylglycerols, multivalent membrane modulators. *Chem Phys Lipids* 148: 1–25.
- Preiss J, Loomis CR, Bishop WR, Stein R, Niedel JE, et al. (1986) Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. *J Biol Chem* 261: 8597–8600.
- Takuwa N, Takuwa Y, Rasmussen H (1987) A tumour promoter, 12-O-tetradecanoylphorbol 13-acetate, increases cellular 1,2-diacylglycerol content through a mechanism other than phosphoinositide hydrolysis in Swiss-mouse 3T3 fibroblasts. *Biochem J* 243: 647–653.
- Sanchez-Pinera P, Micol V, Corbalan-Garcia S, Gomez-Fernandez JC (1999) A comparative study of the activation of protein kinase C alpha by different diacylglycerol isomers. *Biochem J* 337 (Pt 3): 387–395.
- Ogita K, Ono Y, Kikkawa U, Nishizuka Y (1991) Expression, separation, and assay of protein kinase C subspecies. *Methods Enzymol* 200: 228–234.
- Wooten MW, Vandenplas M, Nel AE (1987) Rapid purification of protein kinase C from rat brain. A novel method employing protamine-agarose affinity column chromatography. *Eur J Biochem* 164: 461–467.
- Bolen EJ, Sando JJ (1992) Effect of phospholipid unsaturation on protein kinase C activation. *Biochemistry* 31: 5945–5951.
- Takai Y, Kishimoto A, Iwasa Y, Kawahara Y, Mori T, et al. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J Biol Chem* 254: 3692–3695.
- Medkova M, Cho W (1998) Differential membrane-binding and activation mechanisms of protein kinase C-alpha and -epsilon. *Biochemistry* 37: 4892–4900.
- Corbin JA, Evans JH, Landgraf KE, Falke JJ (2007) Mechanism of specific membrane targeting by C2 domains: localized pools of target lipids enhance Ca²⁺ affinity. *Biochemistry* 46: 4322–4336.
- Torrecillas A, Corbalan-Garcia S, Gomez-Fernandez JC (2004) An infrared spectroscopic study of the secondary structure of protein kinase C alpha and its thermal denaturation. *Biochemistry* 43: 2332–2344.
- Corbalan-Garcia S, Gomez-Fernandez JC (2006) Protein kinase C regulatory domains: The art of decoding many different signals in membranes. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1761: 633–654.
- Oancea E, Meyer T (1998) Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell* 95: 307–318.
- Corbalan-Garcia S, Rodriguez-Alfaro JA, Gomez-Fernandez JC (1999) Determination of the calcium-binding sites of the C2 domain of protein kinase C alpha that are critical for its translocation to the plasma membrane. *Biochemical Journal* 337: 513–521.
- Conesa-Zamora P, Gomez-Fernandez JC, Corbalan-Garcia S (2000) The C2 domain of protein kinase calpha is directly involved in the diacylglycerol-dependent binding of the C1 domain to the membrane. *Biochim Biophys Acta* 1487: 246–254.
- Garcia-Garcia J, Corbalan-Garcia S, Gomez-Fernandez JC (1999) Effect of calcium and phosphatidic acid binding on the C2 domain of PKC alpha as studied by Fourier transform infrared spectroscopy. *Biochemistry* 38: 9667–9675.

41. Bolsover SR, Gomez-Fernandez JC, Corbalan-Garcia S (2003) Role of the Ca²⁺/phosphatidylserine binding region of the C2 domain in the translocation of protein kinase C α to the plasma membrane. *J Biol Chem* 278: 10282–10290.
42. Nalefski EA, Newton AC (2003) Use of stopped-flow fluorescence spectroscopy to measure rapid membrane binding by protein kinase C. *Methods Mol Biol* 233: 115–128.
43. Feng X, Becker KP, Stribling SD, Peters KG, Hannun YA (2000) Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation. *J Biol Chem* 275: 17024–17034.
44. Ganong BR, Loomis CR, Hannun YA, Bell RM (1986) Specificity and mechanism of protein kinase C activation by sn-1,2-diacylglycerols. *Proc Natl Acad Sci U S A* 83: 1184–1188.
45. Mosior M, Newton AC (1998) Mechanism of the apparent cooperativity in the interaction of protein kinase C with phosphatidylserine. *Biochemistry* 37: 17271–17279.
46. Hannun YA, Loomis CR, Bell RM (1986) Protein kinase C activation in mixed micelles. Mechanistic implications of phospholipid, diacylglycerol, and calcium interdependencies. *J Biol Chem* 261: 7184–7190.
47. Hannun YA, Bell RM (1988) Aminoacridines, potent inhibitors of protein kinase C. *J Biol Chem* 263: 5124–5131.
48. Hannun YA, Loomis CR, Merrill AH Jr, Bell RM (1986) Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J Biol Chem* 261: 12604–12609.
49. Bazzi MD, Nelsestuen GL (1987) Role of substrate in determining the phospholipid specificity of protein kinase C activation. *Biochemistry* 26: 5002–5008.
50. Orellana A, Hidalgo PC, Morales MN, Mezzano D, Bronfman M (1990) Palmitoyl-CoA and the acyl-CoA thioester of the carcinogenic peroxisome-proliferator ciprofibrate potentiate diacylglycerol-activated protein kinase C by decreasing the phosphatidylserine requirement of the enzyme. *Eur J Biochem* 190: 57–61.
51. Bittova L, Stahelin RV, Cho W (2001) Roles of ionic residues of the C1 domain in protein kinase C α activation and the origin of phosphatidylserine specificity. *J Biol Chem* 276: 4218–4226.
52. Sandermann H Jr, Duncan TM (1991) Lipid-dependent membrane enzymes. Kinetic modelling of the activation of protein kinase C by phosphatidylserine. *Biochim Biophys Acta* 1069: 235–240.
53. Ausili A, Corbalan-Garcia S, Gomez-Fernandez JC, Marsh D (2011) Membrane docking of the C2 domain from protein kinase C α as seen by polarized ATR-IR. The role of PIP. *Biochim Biophys Acta* 1808: 684–695.
54. Sanchez-Bautista S, Marin-Vicente C, Gomez-Fernandez JC, Corbalan-Garcia S (2006) The C2 domain of PKC α is a Ca²⁺-dependent PtdIns(4,5)P₂ sensing domain: a new insight into an old pathway. *J Mol Biol* 362: 901–914.
55. Leonard TA, Rozycki B, Saidi LF, Hummer G, Hurley JH (2011) Crystal structure and allosteric activation of protein kinase C β II. *Cell* 144: 55–66.
56. Ananthanarayanan B, Stahelin RV, Digma MA, Cho W (2003) Activation mechanisms of conventional protein kinase C isoforms are determined by the ligand affinity and conformational flexibility of their C1 domains. *J Biol Chem* 278: 46886–46894.
57. Sanchez-Bautista S, Corbalan-Garcia S, Perez-Lara A, Gomez-Fernandez JC (2009) A comparison of the membrane binding properties of C1B domains of PKC γ , PKC δ , and PKC ϵ . *Biophys J* 96: 3638–3647.