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The membrane binding kinetics of full-length PKCα is determined by membrane lipid composition

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

Instituto Murciano de Investigaciones Biomédicas, Regional Campus of International Excellence "Campus Mare Nostrum". Departamento de Bioquímica y Biología Molecular-A, Facultad de Veterinaria, Universidad de Murcia, Apartado de Correos 4021, E-30080-Murcia, Spain

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ABSTRACT

Protein kinase Cα (PKCα) is activated by its translocation to the membrane. Activity assays show the importance of PIP_2 in determining the specific activity of this enzyme. A FRET stopped flow fluorescence study was carried out to monitor the rapid kinetics of protein binding to model membranes containing POPC/POPS/DOG and eventually PIP_2. The results best fitted a binding mechanism in which protein bound to the membrane following a two-phase mechanism with a first bimolecular reaction followed by a slow unimolecular reaction. In the absence of PIP_2, the rapid protein binding rate was especially dependent on POPS concentration. Formation of the slow high affinity complex during the second phase seems to involve specific interactions with POPS and DOG since it is only sensitive to changes within relatively low concentration ranges of these lipids. Both the association and dissociation rate constants fell in the presence of PIP_2. We propose a model in which PKCα binds to the membranes via a two-step mechanism consisting of the rapid membrane initial recruitment of PKCα driven by interactions with POPS and/or PIP_2 although interactions with DOG are involved too. PKCα searches on the lipid bilayer in two dimensions to establish interactions with its specific ligands.

1. Introduction

PKCα 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 upon cell stimulation is the hydrolysis of PIP_2 by phospholipase C (PLC) to produce diacylglycerol and IP_3, which activate protein kinase C for sustained cellular responses [2]. However, it has been shown that PIP_2 may also activate PKCα by directly binding to a specific site [3–6], probably a polylysine site located in a β-groove and which can be considered a specific site for PIP_2 [7], although it may also bind to other molecules like phosphatidylserine or phosphatidic acid [8] or even retinoic acid [9]. It has been clearly shown that PIP_2 is important for sustaining PKCα translocation to the membrane and for prolonging this translocation [5,10].

The isolated C2 domain from PKCβII was previously studied by rapid kinetic techniques with respect to its binding to model membranes [11], and in this study special attention was paid to changing the Ca^{2+} concentration. In the same study, the binding of full-length PKCβII was studied with respect to Ca^{2+} concentration. Corbin et al. [12] also used stopped-flow to study the binding of a C2 domain, in this case that of PKCα, to model membranes. They also studied how the binding of isolated C2 domain to model membranes depends on Ca^{2+} concentration, but also on the interplay between Ca^{2+} concentration and the phospholipid targets located in the membrane, namely phosphatidylserine and PIP_2.

In this paper we use highly purified full-length PKCα to extend previous papers carried out with isolated domains, and perform a binding kinetics study to model membranes, in which the concentrations of POPS and DOG, both in the presence and in the absence of PIP_2 are varied. Our results indicate that PKCα binds rapidly to the membrane, preferentially interacting with phosphatidylserine and with diacylglycerol. In a slower phase, it searches the membrane for specific ligands. Both the association and dissociation rate constants decreased in the presence of PIP_2.

Abbreviations: PKC, Protein kinase C; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PIP_2, Phosphatidylinositol 4,5-bisphosphate; DOG, 1,2-Dioleoyl-*sn*-glycerol; IP_3, Inositol 1,4,5-trisphosphate

* Corresponding author. Tel.: +34 868884766.
E-mail address: jcgomez@um.es (J.C. Gómez-Fernández).

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2. Materials and methods

2.1. 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 (PIP_2) and 1,2-*sn*-dioleoylglycerol (DOG) were purchased from Avanti Polar Lipids (Alabaster, ALA, U.S.A.). *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dDHPE) was from Invitrogen (Barcelona, Spain) and all other chemicals were highly pure from Sigma Chemical Co. (Madrid, Spain).

2.2. Expression and purification of protein kinase Cα

A 0.5 l scale culture of Sf9 insect cells at 2.1×10^6 cells/ml was infected with the recombinant baculovirus. The full length cDNA for porcine PKCR was kindly provided by Dr. Robert M. Bell (Duke University Medical Center, Durham, NC). PKCR was expressed in Sf9 (*Spodoptera frugiperda*) insect cells and the protein was purified as described previously [13–15]. 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 μM EGTA, 50 mM NaF, 100 μM $NaVO_3$, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin and 10 mM benzamidine. The pellet was disrupted by sonication (6×10 s) and the resulting lysate was centrifuged at 15,000 rpm for 20 min. The supernatant was applied to a 1 ml His-Gravi Trap™® column (Amersham Biosciences, Uppsala, Sweden) 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 Cα from a His-Gravi Trap™® 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 in a hydrophobic exchange chromatography, directly applying it to a SOURCE 15PHE 4.6/100 PE ® column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with 25 mM 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 PKCα 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.

2.3. 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 (PIP_2) 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).

The vesicle concentration ([v]) was calculated assuming 90,000 phospholipid molecules per vesicle [11,16].

2.4. Fluorescence spectroscopy of transient kinetic processes

Kinetic measurements were carried out on an Applied Photophysics Π^*-180 stopped-flow fluorescence spectrophotometer (Leatherhead, U.K.). Protein-to-membrane fluorescence resonance energy transfer (FRET) was monitored using a 280 nm excitation light and by recording dansyl emission through a 475 nm high-pass filter. Rapid injections of large unilamellar phospholipid vesicles (always in excess of large unilamellar phospholipid vesicles to ensure pseudo first-order conditions) and PKCα (0.2 μM) were carried out. For each time course, five traces were collected, averaged, and subjected to nonlinear, least-squares curve fitting using OriginPro 7.5.

Fluorescence time courses were analyzed with exponential equations of the general form

$$F(t)F_0 + \sum_{i=1}^{n} A_{\mathrm{obs}(i)} \mathrm{e}^{-k_{\mathrm{obs}(i)}t} \tag{1}$$

where $F(t)$ represents the observed fluorescence at time t, F_0 is a fluorescence offset representing the final fluorescence, and $A_{\mathrm{obs}(i)}$ represents the amplitude and $k_{\mathrm{obs}(i)}$ the observed rate constant for the ith of n phases. Observed rate constants and amplitudes were subjected to nonlinear, least-squares curve fitting using Origin 7.5, as described by Nalefski and Newton [11].

The total observed amplitudes of the slow and fast phases were plotted as a function of vesicle concentration and fitted with the hyperbolic equation

$$A_{\mathrm{obs}} = \mathrm{A}_{\mathrm{max}}\left(\frac{[\mathrm{v}]}{[\mathrm{v}] + \mathrm{K}_{\mathrm{d}}^{\mathrm{obs}}}\right) \tag{2}$$

where $\mathrm{A}_{\mathrm{max}}$ represents the calculated maximal amplitude and $\mathrm{K}_{\mathrm{d}}^{\mathrm{obs}}$ represents the observed vesicle dissociation constant.

For the biphasic time courses of PKCα binding to vesicles, the $k_{\mathrm{obs}(1)}$ of the fast phase was plotted as a function of [v] and fitted to the linear equation

$$k_{\mathrm{obs}(1)} = k_1[\mathrm{v}] + C \tag{3}$$

where k_1 represents the apparent second-order rate constant for the bimolecular step and C is a constant equal to the y-intercept. The y-intercept represents the sum $k_{-1}+k_2+k_{-2}$, where k_{-1} equals the apparent dissociation rate constant for the bimolecular step and k_2+k_{-2} is the sum of the forward and reverse rate constants for a first-order transition step. For the slow phase of PKCα binding to vesicles, $k_{\mathrm{obs}(2)}$ was plotted as a function of [v] and fitted to a hyperbolic equation

$$k_{\mathrm{obs}(2)} = k_{\mathrm{max}(2)}\left(\frac{[\mathrm{v}]}{[\mathrm{v}] + C}\right) \tag{4}$$

where C is a constant and $k_{\mathrm{max}(2)}$ represents the calculated asymptote, which is equal to the sum k_2+k_{-2}.

The observed rate constants $k_{\mathrm{off}(1)}$ and $k_{\mathrm{off}(2)}$ for the biphasic membrane dissociation of PKCα were measured by rapid 10-fold dilution experiments, diluting the PKCα-vesicle complexes formed by the binding of large unilamellar phospholipid vesicles at a concentration of 500 μM and PKCα at a concentration of 0.2 μM. The value taken for $k_{\mathrm{off}(1)}$ and $k_{\mathrm{off}(2)}$ were derived directly from $k_{\mathrm{obs}(1)}$ and $k_{\mathrm{obs}(2)}$, respectively. The value taken for k_{off} was derived from the average of 30 runs.

2.5. Enzymatic activity assay

Enzymatic activity was assayed using a technique described previously [17], in which the incorporation of radioactive phosphate [γ-^{32}P] to kinase substrate (histone III-S) was measured.

Lipids in organic solutions were mixed in the desired proportions and dried under a stream of N_2, removing the last traces of organic solvent by keeping the samples under vacuum for at least 4 h. Large unilamellar