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The C1B domains of novel PKCε and PKCη have a higher membrane binding affinity than those of the also novel PKCδ and PKCθ

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ABSTRACT

The C1 domains of novel PKCs mediate the diacylglycerol-dependent translocation of these enzymes. The four different C1B domains of novel PKCs (δ, ε, θ and η) were studied, together with different lipid mixtures containing acidic phospholipids and diacylglycerol or phorbol ester. The results show that either in the presence or in the absence of diacylglycerol, C1Bε and C1Bη exhibit a substantially higher propensity to bind to vesicles containing negatively charged phospholipids than C1Bδ and C1Bθ. The observed differences between the C1B domains of novel PKCs (in two groups of two each) were also evident in RBL-2H3 cells and it was found that, as with model membranes, in which C1Bε and C1Bη could be translocated to membranes by the addition of a soluble phosphatidic acid without diacylglycerol or phorbol ester, C1Bδ and C1Bθ were not translocated when soluble phosphatidic acid was added, and diacylglycerol was required to achieve a detectable binding to cell membranes. It is concluded that two different subfamilies of novel PKCs can be established with respect to their propensity to bind to the cell membrane and that these peculiarities in recognizing lipids may explain why these isoenzymes are specialized in responding to different triggering signals and bind to different cell membranes.

1. Introduction

PKC (protein kinase C) is a large family of phospholipid-dependent serine/threonine kinases, which are activated by many extracellular signals, and which play a critical role in several signaling pathways in the cell. Mammalian isoenzymes have been grouped into three subfamilies according to their enzymatic properties. The first group, called classical or conventional isoenzymes (cPKCs), 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 Ca^{2+} and acidic phospholipids, which interact with the C2 domain, and by DAG (diacylglycerol), which interacts with the C1 domain [1,2]. Classical PKC isoenzymes are translocated to the plasma membrane of mammalian cells when the Ca^{2+} concentration is increased in the cytoplasm through the bridging made by this cation between the C2 domain and negatively charged phospholipids in the membrane (with preference phosphatidylserine) [3], and also by interaction with PIP_2 in the membrane through a second site [4–7], followed by C1 domain–diacylglycerol interactions [2,8,9]. In contrast, the enzymes of the novel PKC subfamily (δ, ε, θ and η) do not bind Ca^{2+} and are activated through diacylglycerol or phorbol esters binding to the C1 domain [1], although the C2 domain also plays a role through interactions with ligands like phosphatidic acid [10,11].

Both classical and novel PKCs possess two different C1 domains, which form a tandem in the regulatory domain, C1A and C1B. A large number of experimental observations suggest that both domains are functionally dissimilar, as is evident for example, from their different affinities for diacylglycerols or phorbol esters [2,12–15]. The affinity of C1A and C1B for these ligands changes depending on the isoenzyme in question. For example, it has been described that whereas the C1A domain of PKCα shows a much higher DAG affinity than the C1B domain, furthermore, the C1A has also a higher affinity for phorbol esters than the C1B but affinities are not so different in this case [13,16]. In the case of other classical isoenzymes, like β [13] and γ [12,13,16,17] differences are not big for diacylglycerol or phorbol ester affinities between the C1A and C1B domain affinities.

For PKCδ, conflicting results have been published, one group [13] claiming that C1B has a much higher binding affinity for diacylglycerol than C1A, while another group [18] maintains that the opposite is true. PKCε is similar to PKCγ in that both C1A and C1B domains are involved in membrane binding and activation, although the C1A domain has about a 3-fold higher DAG affinity than the C1B domain [19]. With

Abbreviations: OG-PE, N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; DOG, 1,2-*sn*-dioleoylglycerol; ECFP, enhanced cyan fluorescent protein; SAG, 1-stearoyl-oleoyl-*sn*-glycerol; DPG, 1,2-*sn*-dipalmitoylglycerol; DOcPA, 1,2-dioctanoyl-*sn*-phosphatidic acid; POPG, 1-palmitoyl-2-oleoyl-*sn* phosphatidylglycerol; POPA, 1-palmitoyl-2-oleoyl-*sn*-phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-phoshatidylserine; DOcG, 1,2-dioctanoylglycerol; K_D, apparent equilibrium dissociation constant

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respect to the other new isoenzymes, fewer studies have been carried out, but it seems that in both θ and η the C1B domain has a higher affinity than the C1A domain for phorbol esters [13,17].

It has long been known that phosphatidylserine enhances the membrane affinity and activity of PKCs [20], although it has also been claimed that the phosphatidylserine dependence may vary significantly among PKC isoforms [16,21]. Among conventional PKCs, PKCα [21] and PKCβII [22] prefer PS to phosphatidylglycerol, whereas PKCγ shows little preference between phosphatidylserine and phosphatidylglycerol [16]. Among novel PKCs, PKCδ shows a certain degree of phosphatidylserine selectivity [18], whereas PKCε does not [19,21].

Nevertheless, it should be taken into account that both the C1 and C2 domains bind anionic phospholipids, and so it is difficult to use whole-enzyme activity to detect from which domain the observed specificity arises. For this reason, it is important to study the isolated domains. In the case of the C1 domain, only a limited number of studies have been carried out for this purpose, and, among the findings, it may be mentioned that the C1B domain of PKCβII (in the presence of diacylglycerol) has a dissociation constant of 780 μM in the presence of phosphatidylserine and 1690 μM in the presence of phosphatidylglycerol [23]. However, when a Tyr residue was mutated to Trp, the C1B domain showed a dissociation constant of 24 μM for a membrane containing phosphatidylserine and 130 μM for a membrane containing phosphatidylglycerol, i.e., although the constant was considerably reduced, the preference was not a pronounced preference for phosphatidylserine [24]. In the case of C1Bδ, it was clearly shown that the preference was for phosphatidylserine (K_D of 35 μM) rather than for phosphatidylglycerol (K_D of 700 μM) [23].

A study was carried out using isolated C1B domains of three different PKCs (the classical γ, and the novel δ and ε) binding to liposomes of different compositions, using three different DAGs (DOG, SAG and DPG) and three different anionic phospholipids (phosphatidylserine, phosphatidic acid and phosphatidylglycerol) to prepare the model vesicles. C1Bε was found to have the highest binding affinity to vesicles containing phosphatidic acid as acidic phospholipid, and DOG or SAG as diacylglycerol. In general, DOG and SAG led to a higher membrane binding affinity than DPG in all the C1B domains [25]. In this paper we amplify this study by comparing, side by side, the four C1B domains from novel PKCs, and their affinities for membranes containing different negatively charged phospholipids. Two groups could be established, one with a lower K_D for model membranes, constituted by C1Bε and C1Bη, and another formed by C1Bδ and C1Bθ which show substantially higher K_D. The lowest K_D in the case of C1Bε and C1Bη was observed for membranes containing POPA. These differences in membrane affinities were confirmed by expressing the four domains in RBL-2H3 cells and triggering the translocation to the plasma membrane by adding a soluble phosphatidic acid or a soluble diacylglycerol.

2. Materials and methods

2.1. Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Oregon Green 488-dihexadecanoylphosphatidylethanolamine (OG-PE) was obtained from Invitrogen, Barcelona, Spain. Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Co. (Madrid, Spain).

2.2. Construction of the expression plasmids

C-terminal fusions of isolated C1 domains were generated by inserting cDNAs into the multiple cloning site of the pECFP vector modified and described by [25].

Briefly, cDNAs encoding C1B domains of PKCθ, δ and ε were amplified by PCR using the following primers:

C1Bθ: 5′ CGGAATTCATCGCTTTAAAGTGTAT
C1Bθ: 3′ CCCAAGCTTTCAGCACAGGTTCGCCAC
C1Bδ: 5′ TATAAGCTTGACATGCCTCACCGA
C1Bδ: 3′ GACACACCATAGTTGACTCCTAGGAA
C1Bε: 5′ CCGAAGCTTAACATGCCCCACAAG
C1Bε: 3′ GTTAACACCCCACCTGACTGGGCCCTAAA

C1Bε was digested with HindIII/XmaI, C1Bδ was digested with HindIII/BamHI and C1Bθ was digested with EcoRI/ BamHI. C1Bη was synthesized and cloned into the EcoRI and BamHI sites of the plasmid vector pUC57 by GenScript Corporation (Piscataway, New Jersey, USA). The resulting fragments were ligated with their corresponding digested vectors to generate the different fusion constructs.

All constructs were confirmed by DNA sequencing in the Research and Development Support Center (CAID), Universidad de Murcia (Spain).

2.3. Cell culture and transfections

HEK 293 cells (ECACC, European Collection of Cell Cultures) were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with 2 μg DNA/6 cm plate using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. The cells were lysed 24 h after transfection in ice-cold hypotonic buffer (10 mM Tris pH 7.4, 10 mM NaF, 1 mM orthovanadate, 1 mM PMSF and 10 μg/ml each aprotinin and leupeptin) and incubated on ice (20 min). Cells were lysed by 15 passages through a 30-gauge needle; lysates were centrifuged (15,000 ×*g*, 15 min) to remove nuclei and cell debris. Supernatants were collected and used in the fluorescence experiments.

Rat basophilic leukemia (RBL-2H3) cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in a growth medium of Dulbecco's modified Eagle's Medium supplemented with 15% (vol/vol) fetal calf serum. Cells were prepared for confocal microscopy as described by [10]. Basically, harvested cells were resuspended in electroporation buffer (120 mM NaCl, 5.5 mM KCl, 2.8 mM $MgCl_2$, 25 mM glucose, 20 mM HEPES, pH 7.2) and 30 μg of cDNA. The cells were electroporated in a GenePulser (Bio-Rad, Hercules, CA) with one 200 V/10 ms square wave pulse and immediately placed on ice for 5 min before being plated on glass coverslips and incubated at 37 °C for 4–6 h, after which the growth medium was renewed. RBL cells were used 16–24 h later after priming overnight with 500 ng/ml IgE-anti-dinitrophenyl (mouse monoclonal; Sigma-Aldrich Quimica, S.A., Madrid, Spain) and stimulation was performed using 2 μg/ml dinitrophenyl-human serum albumin [DNP-HSA]. The coverslips were washed with 3 ml of extracellular buffer HBS (120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES pH 7.2). All added substances were dissolved or diluted in HBS. 1,2-Dioctanoylglycerol (DOcG) and DOcPA were dissolved in dimethyl sulfoxide and diluted to the final concentration with extracellular buffer shortly before the experiment. During the experiment, the cells were not exposed to dimethyl sulfoxide concentrations higher than 1%. All the experiments were carried out at room temperature and, unless otherwise stated, on at least four different occasions. In each experiment, recordings were obtained from two to six cells.

2.4. Binding of C1B-ECFP domains to lipid vesicles

Binding experiments were carried out using small unilamellar vesicles obtained by sonication of multilamellar vesicles, which were prepared by desiccating mixtures of chloroform solutions of lipids in the suitable proportions and extensive vortexing. Lipid mixtures contained phospholipids POPC/POPX/OG-PE, in the desired proportions, where POPX stands for POPS, POPA or POPG and diacylglycerols (DOG) or phorbol esters (PMA). Binding experiments were performed at 25 °C, using a FluroMax-3 (Jobin Yvon, Horiba, Edison, NJ, USA), with a 20 mM Hepes (pH 7.4) and 100 mM KCl buffer. The excitation and emission windows

were set to 3 and 4 nm, respectively. To normalize the amount of protein between different experiments, cell lysates were added to the fluorescence cuvette to reach the same starting ECFP fluorescence intensity. FRET was measured as the fluorescence variation of ECFP as a function of lipid concentration. The intensity of ECFP fluorescence was measured using an excitation wavelength of 433 nm and collected at 473 nm. To correct for the fluorescence attenuation produced by the lipidic vesicles, a control was measured in which vesicles containing only POPC and OG-PE were added to a cuvette with C1B-ECFP, while the values obtained were subtracted from these of the above experiments. Additionally, the dilution effect produced by the addition of vesicles was corrected by subtracting the values obtained after adding the same volumes of buffer without lipid vesicles to a cuvette containing the C1B domain. The mean values of three different experiments are shown.

Equilibrium binding data were best-fitted using the Hill Eq. (1):

$$\Delta F = \Delta F_{max}\left(X^H/{K_D}^H + X^H\right) \quad (1)$$

where ΔF_{max} represents the calculated maximal fluorescence change (normalized to unity to simplify graphical representation), H is the Hill coefficient, X represents the free phospholipid concentration corrected for the leaflet effect (for phospholipid titrations) and K_D represents the apparent equilibrium dissociation constant for lipid binding and corresponds to the inverse of the affinity constant. Note that the amount of protein added is very low since it is comes from the cell lysates of C1B-overexpressing HEK293 cells, and from the first lipid additions when the [protein] ≪ [lipid]. Therefore, free concentrations of phospholipid can be assumed to be approximately the same as the total lipid concentrations.

2.5. Confocal microscopy

Cells expressing various C1B-ECFP constructs were washed with HBS and examined using a TCS SP confocal system (Leica, Heidelberg, Germany) with a Nikon PLAN APO-CS 63 × 1.2 numerical aperture water immersion objective.

Confocal images were obtained by excitation at 405 nm and emission wavelengths at 470–475 nm for CFP. During imaging, cells were stimulated with DNP-HSA, DOcG or DOcPA. Series of 60–120 confocal images were recorded for each experiment at time intervals of 3 s.

2.6. Image analysis

Background was subtracted from the images before the calculations were performed. The time series were analyzed using Image J NIH software (http://rsb.info.nih.gov/ij/, 1997–2003). An individual analysis of protein translocation for each cell was performed by tracing a line intensity profile across the cell [26]. The relative increase in the amount of enzyme localized in the plasma membrane for each time point was calculated by using the ratio R (Imb Icyt)/Icyt, where Imb is the fluorescence intensity at the plasma membrane and Icyt is the average cytosolic fluorescence intensity. Mean values are given SE of the mean.

3. Results

The aim of this work was to study the membrane binding affinity of C1 domains of novel PKCs for different anionic phospholipids in the presence or absence of DOG or PMA. C1B domains from PKCδ, PKCε, PKCη and PKCθ were used.

Since these C1B domains lack suitable residues to induce fluorescence energy transfer between a membrane probe and Trp residues, a FRET method was used, in which the acceptor was phosphatidylethanolamine labeled with Oregon Green-488 (OG-PE) located in the membrane, and the donor was the C1 domain fused to enhanced cyan fluorescent protein (ECFP). In a second phase the membrane translocation of these C1B domains fused to a fluorescent protein was examined in RBL-2H3.

3.1. The influence of acidic phospholipids on the binding of C1B domains to membrane in the presence of DOG

Using the FRET method mentioned above, the binding of C1B domains to small unilamellar vesicles containing different lipid compositions was studied. Fig. 1 shows the binding of the C1Bδ domain to increasing concentrations of POPC/POPX/DOG vesicles, where POPX stands for POPS (Fig. 1A), POPG (Fig. 1B) or POPA (Fig. 1C). In this assay, the effect of increasing the relative percentage of acidic phospholipid on C1B membrane binding was also studied. The data were analyzed and best-fitted to the Hill model. The diacylglycerol (DOG) concentration was kept constant at 5 mol% in all the following cases.

C1Bδ (Fig. 1, Table 1) showed the highest binding affinity for membranes when POPS was present although all the values were within the same order of magnitude (at 40 mol% of anionic phospholipids, the K_D was 11.57 ± 0.2 μM for POPS compared to 19.55 ± 0.4 μM for POPA and 37.54 ± 0.7 μM for POPG).

In the case of C1Bθ (Fig. 1, Table 1), the lowest K_D corresponded to membranes incorporating POPA (Fig. 1F), followed by those with POPS (Fig. 1D) and finally those with POPG (Fig. 1E), although all of them were in the same order of magnitude (at 40 mol% of the anionic phospholipids, the K_D was 6.78 ± 1.19 μM for POPA, 11.99 ± 1.79 for POPS and 22.64 ± 1.01 μM for POPG).

When the binding of C1Bε was studied, it was observed (Fig. 2 and Table 1) that the extent of binding was much higher than with the two previously mentioned isoenzymes. In this case, the highest binding affinity was very clearly for POPA (Fig. 2C), followed by POPS (Fig, 2A) and POPG (Fig. 2B and Table 1) (at 40 mol% of the anionic phospholipids, the K_D was 0.27 ± 0.05 μM for POPA, 1.01 ± 0.19 μM for POPS and 3.01 ± 0.45 μM for POPG).

In the case of C1Bη, binding affinities were more similar to those observed for the ε isoenzyme than to those observed for the δ and θ isoenzymes. As with the PKC-C1Bε, the highest binding affinity was observed for POPA (Fig. 2F), followed by POPS (Fig. 2D) and POPG (Fig. 2E) (at 40 mol% of the anionic phospholipids, the K_D was 0.40 ± 0.08 μM for POPA, 0.98 ± 0.81 for POPS and 1.08 ± 0.15 μM for POPG) (Table 1).

An interesting finding was the important role played by anionic phospholipids in the binding of C1B domains to the membranes (Table 1). As the contents of the anionic phospholipids increased from 5 to 40 mol%, the membrane binding affinity increased up to one order of magnitude at a constant DOG concentration (5 mol%). See, for example, the case of C1Bε with POPA, with K_D 2.74 ± 0.48 μM at 5 mol% and 0.27 ± 0.05 μM at 40 mol%; or that of C1Bη with POPA, when the increase was from K_D of 3.63 ± 1.15 μM at 5 mol% and 0.40 ± 0.08 μM at 40 mol% (Table 1). These results confirm that not only is diacylglycerol important for the binding of C1 domains to the membrane, but that anionic phospholipids are also important.

Another interesting result was that there were substantial differences in the binding affinity of the domains studied. The highest binding affinities were observed in C1Bε (Fig. 2 and Table 1) for the three anionic phospholipids, the increase being about 25-fold for 40 mol% of POPA (K_D values of 0.27 ± 0.05 μM for C1Bε compared with 19.55 ± 0.4 μM for C1Bδ and 6.78 ± 1.19 μM for C1Bθ). More similar were the membrane binding affinities of isoenzyme δ compared with θ and that of ε compared with η. Increases of up to 10-fold were also observed in the presence of 40 mol% POPS (K_D values of 1.01 ± 0.19 μM for C1Bε compared to 11.99 ± 1.79 μM for C1Bθ and 11.57 ± 0.2 μM for C1Bδ) and up to 37-fold when 40 mol% POPG was present in the membrane (K_D values of 1.08 ± 0.15 μM for C1Bη and 3.01 ± 0.45 for C1Bε compared with 22.64 ± 1.01 μM for C1Bθ and 37.54 ± 0.7 μM for C1Bδ).

Thus, in general, similar membrane binding affinities were observed for C1Bθ and C1Bδ and, on the other hand, for C1Bε and C1Bη.

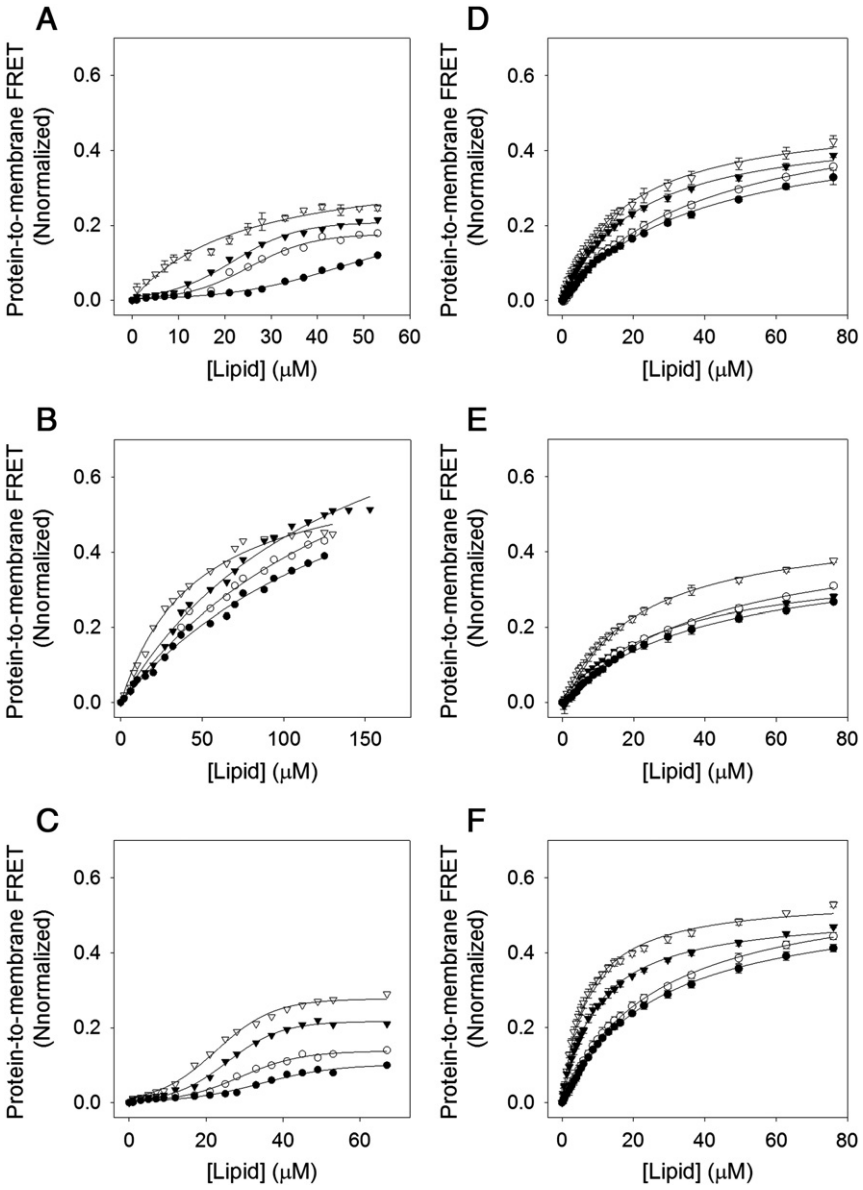


Fig. 1. Binding of C1Bδ and C1Bθ to lipid vesicles in the presence of DOG. Binding of C1Bδ-ECFP domain (A, B and C) and C1Bθ-ECFP domain (D, E and F) to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OG-PE in molar ratios of 90:5:5 (•), 85:10:5 (o), 75:20:5 (▼), and 55:40:5 (▽). POPX was POPS (A and D), POPG (B and E) or POPA (C and F). DOG was present in all cases at 5 mol%. Normalized FRET values are depicted versus total lipid concentration.

Table 1
Binding parameters of C1B-ECFP domains to phospholipid vesicles.

		C1Bδ		C1Bθ		C1Bε		C1Bη	
	mol%	ΔF_{max} (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)	ΔF_{max} × (%)	K_D (μM)	ΔF_{max} (%)	K_D (μM)
POPS	5	0.12 ± 0.02	40.15 ± 0.4	0.32 ± 0.02	40.25 ± 0.78	0.46 ± 0.01	6.76 ± 0.66	0.45 ± 0.01	6.28 ± 0.63
	10	0.20 ± 0.01	28.22 ± 0.2	0.35 ± 0.01	39.36 ± 0.53	0.46 ± 0.02	3.23 ± 0.62	0.59 ± 0.03	2.55 ± 0.15
	20	0.24 ± 0.02	22.60 ± 0.2	0.38 ± 0.02	20.92 ± 0.49	0.60 ± 0.01	1.91 ± 0.12	0.58 ± 0.02	2.02 ± 0.04
	40	0.30 ± 0.01	11.57 ± 0.2	0.42 ± 0.01	11.99 ± 1.79	0.61 ± 0.03	1.01 ± 0.19	0.61 ± 0.03	0.98 ± 0.81
POPA	5	0.11 ± 0.03	35.33 ± 0.4	0.44 ± 0.01	30.39 ± 1.46	0.54 ± 0.01	2.74 ± 0.48	0.45 ± 0.01	3.63 ± 1.15
	10	0.16 ± 0.02	30.29 ± 0.4	0.44 ± 0.01	26.02 ± 0.93	0.57 ± 0.01	1.69 ± 0.06	0.47 ± 0.01	1.89 ± 0.21
	20	0.21 ± 0.05	20.40 ± 0.8	0.46 ± 0.01	9.66 ± 1.13	0.62 ± 0.01	1.18 ± 0.08	0.43 ± 0.01	1.11 ± 0.03
	40	0.30 ± 0.01	19.55 ± 0.4	0.52 ± 0.01	6.78 ± 1.19	0.65 ± 0.01	0.27 ± 0.05	0.52 ± 0.01	0.40 ± 0.08
POPG	5	0.43 ± 0.01	64.78 ± 0.2	0.26 ± 0.01	42.49 ± 2.89	0.50 ± 0.01	8.03 ± 0.55	0.42 ± 0.01	12.6 ± 0.64
	10	0.43 ± 0.02	52.67 ± 0.2	0.30 ± 0.01	37.74 ± 2.61	0.61 ± 0.01	6.32 ± 0.42	0.44 ± 0.01	5.01 ± 0.54
	20	0.52 ± 0.01	48.29 ± 0.2	0.29 ± 0.01	28.88 ± 2.56	0.64 ± 0.02	4.18 ± 0.45	0.44 ± 0.01	3.71 ± 0.65
	40	0.53 ± 0.01	37.54 ± 0.7	0.37 ± 0.01	22.64 ± 1.01	0.67 ± 0.01	3.01 ± 0.45	0.53 ± 0.03	1.08 ± 0.15

Phospholipid vesicle contained POPC/DOG/POPX/OG-PE (X:5:X:5 mol%), whereas anionic and POPC phospholipids varied as indicated in the table. FRET data were fitted to a Hill equation. Values given as mean ± SE of three independent experiments in triplicates (n = 9).

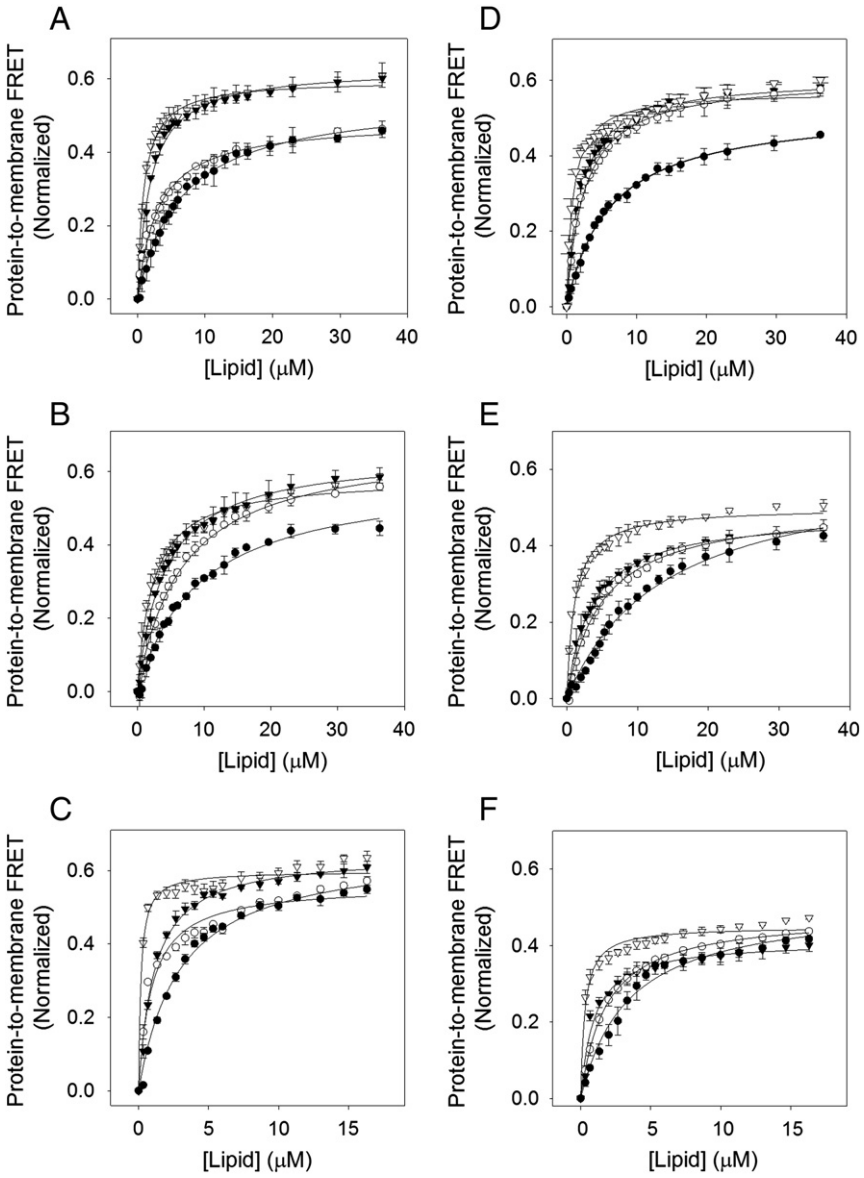


Fig. 2. Binding of C1Bε and C1Bη to lipid vesicles in the presence of DOG. Binding of C1Bε-ECFP domain (A, B and C) and C1Bη-ECFP domain (D, E and F) to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OG-PE in molar ratios of 90:5:5 (•), 85:10:5 (o), 75:20:5 (▼) and 55:40:5 (▽). POPX was POPS (A and D), POPG (B and E), or POPA (C and F). DOG was present in all cases at 5 mol%. Normalized FRET values are depicted versus total lipid concentration.

3.2. The influence of acidic phospholipids on the binding of C1B domain to membrane in the absence of diacylglycerol or in the presence of PMA

The binding dependency of the C1B domains to lipid vesicles was also studied in the total absence of diacylglycerol. Fig. 3 shows a comparison of the four C1B domains studied, in the presence of 20 mol% of each anionic phospholipid. The existence of two groups of C1B domains delimited by their membrane binding affinity is clearly revealed by these results, especially in the presence POPA (Fig. 3C) and POPS (Fig. 3A). The same pattern was found in the presence of POPG (Fig. 3B) although with a less pronounced separation between the two pairs. Table 2 shows that K_D values had a similar order of magnitude for membranes containing any of the anionic phospholipids tested in the case of C1Bδ and C1Bθ, ranging from 32.06 ± 0.46 μM for C1Bδ with POPS to 52.21 ± 0.84 μM for the same C1Bδ with POPG. These figures were much lower in the case of C1Bε (K_D values of 25.43 ± 0.19 μM for POPS, 3.02 ± 0.34 for POPA and 21.61 ± 0.39 for POPG) and for C1Bη (K_D values of 9.48 ± 0.80 μM for POPS, 1.82 ± 0.5 for POPA and 15.98 ± 0.76 for POPG). Note the strongly decreased value in the presence of POPA, indicating that in these cases membrane translocation may already be quite high even without diacylglycerol. The addition of 5 mol% DOG increases membrane affinity in all cases compared with membranes without any diacylglycerol, but especially with C1Bε and C1Bη, revealing once again the higher affinity for membranes of these two domains. Such was the case of C1Bε with POPS (K_D 25.43 ± 0.19 in the absence and 1.91 ± 0.12 μM in the presence of DOG) and with POPG (K_D 21.61 ± 0.39 in the absence and 4.18 ± 0.45 μM in the presence of DOG) and also of C1Bη with POPS (K_D 9.48 ± 0.80 in the absence and 2.02 ± 0.04 μM in the presence of DOG) and POPG (K_D 15.98 ± 0.76 in the absence and 3.71 ± 0.65 μM in the presence of DOG). The decrease in K_D values was less marked in the presence of POPA in the case of C1Bε (from 1.18 ± 0.08 μM in the presence of DOG to 3.02 ± 0.34 in its absence) and for C1Bη (1.11 ± 0.03 μM in the presence and 1.82 ± 0.5 in the absence of DOG).

In the presence of 1 mol% of PMA with respect to the total lipid, however (Fig. 4) membrane binding affinities were similar for all C1B domains

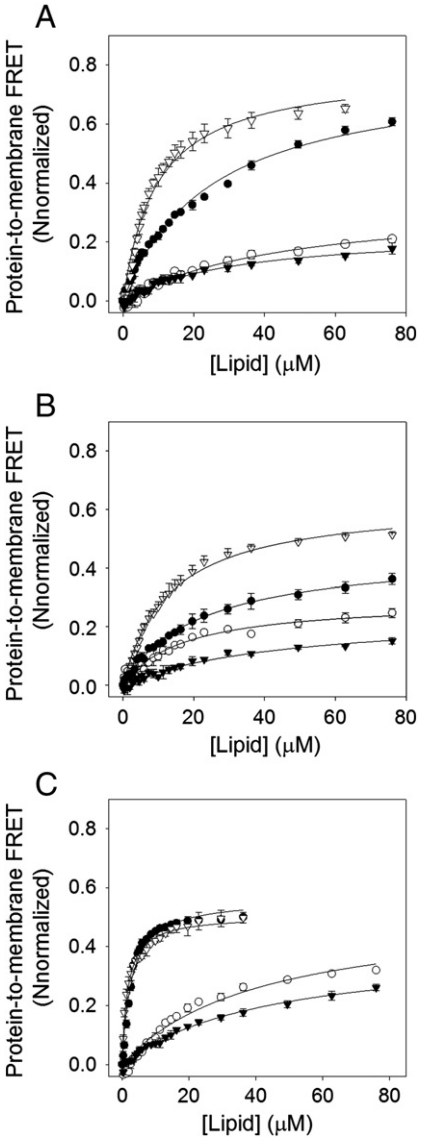


Fig. 3. Binding of C1B domains to lipid vesicles in the absence of DOG. Binding of C1Bθ-ECFP(o), C1Bε-ECFP(•), C1Bη-ECFP(▽) and C1Bδ-ECFP(▼) domains to lipid vesicles, which contained POPC/POPX/OG-PE in molar ratio of 75:20:5. POPX was POPS (A), POPG (B) or POPA (C). Normalized FRET values are depicted versus total lipid concentration.

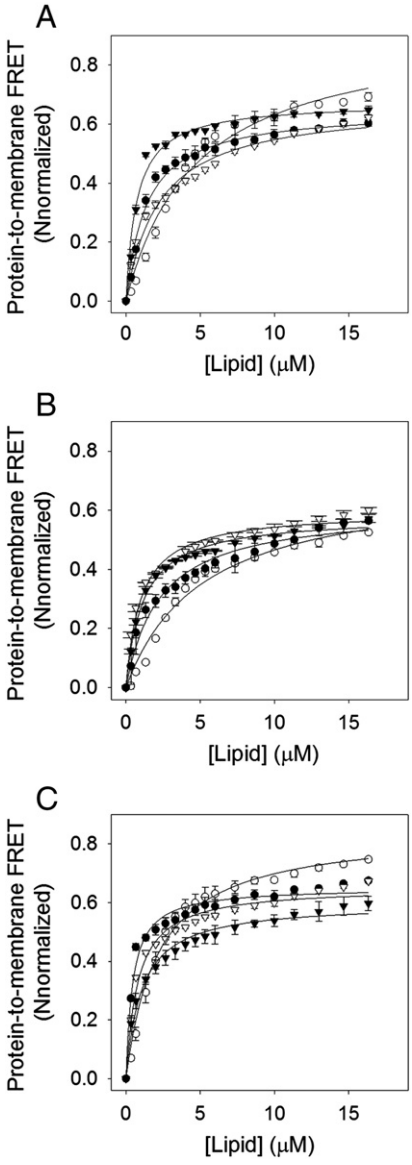


Fig. 4. Binding of C1B domains to lipid vesicles in the presence of PMA. Binding of C1Bθ-ECFP (o), C1Bε-ECFP (•), C1Bη-ECFP (▽), and C1Bδ-ECFP (▼) domains to lipid vesicles contained POPC/POPX/PMA/OG-PE in molar ratio of 74:20:1:5. POPX was POPS (A), POPG (B) or POPA (C). Normalized FRET values are depicted versus total lipid concentration.

studied and for all anionic phospholipids, indicating that this activator was at saturating concentration and that all these domains are able to translocate to the membrane if the concentration of activating molecules is sufficiently high (see also Table 3). Note that the K_D values reached in this case for C1Bε and C1Bη were not much lower than those reached with 5 mol% DOG and even in the presence of POPA (in the total absence of DOG or PMA), as it occurred for C1Bη with POPA (K_D was 1.82 ± 0.5 in the absence of DOG and PMA, but 1.11 ± 0.03 μM in the presence of 5 mol% DOG and 1.01 ± 0.12 in the presence of 1 mol% PMA). This indicates that these C1B domains may reach nearly maximum membrane binding in the presence of POPA alone.

Table 2
Binding parameters of C1B-ECFP domains to phospholipid vesicles.

	mol% DOG	C1Bδ	C1Bθ	C1Bε	C1Bη
		K_D (μM)	K_D (μM)	K_D (μM)	K_D (μM)
POPS	0	32.06 ± 0.46	47.02 ± 0.68	25.43 ± 0.19	9.48 ± 0.80
	5	22.60 ± 0.20	20.92 ± 0.49	1.91 ± 0.12	2.02 ± 0.04
POPA	0	43.65 ± 0.79	41.35 ± 0.88	3.02 ± 0.34	1.82 ± 0.50
	5	20.40 ± 0.83	9.66 ± 1.13	1.18 ± 0.08	1.11 ± 0.03
POPG	0	52.21 ± 0.84	44.94 ± 0.65	21.61 ± 0.39	15.98 ± 0.76
	5	48.29 ± 0.25	28.88 ± 2.56	4.18 ± 0.45	3.71 ± 0.65

Phospholipid vesicle contained 75 mol% POPC, X mol % of DOG, 5 mol% OG-PE, and 20 mol% of the indicated anionic phospholipid. FRET data were fitted to a Hill equation. Values given as mean ± SE of three independent experiments in triplicates (n = 9).

Table 3
Binding parameters of C1B-ECFP domains to phospholipid vesicles.

	Mol (%) PMA	C1Bδ	C1Bθ	C1Bε	C1Bη
		K_D (μM)	K_D (μM)	K_D (μM)	K_D (μM)
POPS	1	0.74 ± 0.06	3.71 ± 0.17	1.39 ± 0.06	0.86 ± 0.44
POPA	1	1.32 ± 0.20	2.14 ± 0.12	0.49 ± 0.07	1.01 ± 0.12
POPG	1	1.46 ± 0.15	5.15 ± 0.12	2.67 ± 0.12	1.14 ± 0.19

Phospholipid vesicle contained 74 mol% POPC, 1 mol% of PMA, 5 mol% OG-PE, and 20 mol% of the indicated anionic phospholipid. FRET data were fitted to a Hill equation. Values given as mean ± SE of three independent experiments in triplicates (n = 9).

Table 4
Plasma membrane translocation parameters in RBL-2H3 cells stimulated with antigen DNP-HSA.

Stimulation 2 μg/ml DNP-HSA	*N* cells	M.L., %	R_{max}	$t_{½}$, *s*
C1Bε-ECFP	62	80	0.62 ± 0.01	19 ± 2
C1Bη-ECFP	77	76	0.59 ± 0.02	23 ± 2
C1Bδ-ECFP	63	61	0.52 ± 0.03	37 ± 2
C1Bθ-ECFP	50	66	0.56 ± 0.02	40 ± 6

RBL2H3 cells were primed with 0.5 μg/ml antiIgE antibody for 16 h and then stimulated with 2 μg/ml DNP-HSA. M.L. is membrane localization and indicates the percentage of cells responding to DNP-HSA stimulation with plasma membrane translocation. R_{max} is the maximal relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

3.3. Membrane translocation of C1B domains in RBL-2H3 cells induced by cell activation and endogenous production of activators

To investigate membrane translocation of the four domains in a *cellular* context we have used RBL-2H3 cells transfected with the different protein C1B-ECFP constructs. These cells may be activated by their stimulation of the RBL-2H3 cells by an antigen as DNP-HSA. The cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in mast or basophilic cells (as the RBL-2H3 ones) stimulates a number of lipid-signaling events, which include the activation of phospholipase Cγ phospho-inositide 3-kinase, and phospholipase D (PLD) [72–74] and many serine/threonine kinases [75–77], including the PKC family among others [78]. Diacylglycerols are generated through this stimulation and also phosphatidic acid (López-Andreo et al. 2003).

The results obtained (Table 4 and Fig. 5A) indicate that the four domains may be translocated to the plasma membrane after the stimulation of the cells but some differences were observed between them. With respect to R_{max} C1Bε and C1Bη present slightly higher values (0.62 ± 0.01 and 0.59 ± 0.02, respectively) than C1Bδ and C1Bθ (0.52 ± 0.03 and 0.56 ± 0.02, respectively) but substantial differences were observed for $t_{½}$ with values remarkably lower for C1Bε and C1Bη (19 ± 2 and 23 ± 2 *s*, respectively) in comparison to C1Bδ and C1Bθ (37 ± 2 and 40 ± 6 *s*, respectively). This means that higher concentrations of activating factors are needed for the second couple of domains and thus a longer time for the membrane translocation is needed, although they are able of doing so, as it was also shown with the model system when PMA was added (Table 3).

3.4. Membrane translocation of C1B domains in RBL-2H3 cells induced by permeable activators

To check the importance of phosphatidic acid and diacylglycerol on the membrane translocation of the four domains and if the lag response observed for C1Bδ and C1Bθ is really due to their need of higher concentrations of activators, water soluble forms of the lipids were used that are known to permeate the membranes, namely phosphatidic acid (DOcPA) and soluble diacylglycerol (DOcG).

Table 5 shows that when a high concentration (20 μg/ml) of DOcPA was added to RBL-2H3 cells membrane translocation was induced in the case of C1Bε and C1Bη (R_{max} 0.44 ± 0.22 and 0.41 ±0.02, respectively). The addition of low concentrations of DOcPA (10 μg/ml) (Fig. 5B and Table 5) led to still detectable membrane translocation (R_{max} 0.33 ± 0.03 and 0.21 ± 0.02, respectively).

On the other hand, the addition of 12 μg/ml of DOcG without DOcPA (Table 5) induced considerable membrane translocation (R_{max} 0.46 ± 0.02 for C1Bε and 0.41 ± 0.01 for C1Bη). Considerable less translocation was observed (Fig. 5C and Table 5) when only 4 μg/ml of DOcG was added (R_{max} 0.29 ± 0.03 for C1Bε and 0.26 ± 0.01 for C1Bη). An additive effect was observed when DOcPA (10 μg/ml) and DOcG (4 μg/ml) were added together (Fig. 5D and Table 5) producing, in this case, a good level of translocation (R_{max} 0.51 ± 0.01 for C1Bε and 0.53 ± 0.03 for C1Bη).

In contrast with the results shown above in RBL-2H3 cells for C1Bε and C1Bη, no translocation was observed for C1Bδ and C1Bθ when the same concentrations of DOcPA were used (Fig. 5E and Table 5) and some degree of translocation was only observed for these domains when high concentrations of DOcG were used (e.g. 12 μg/ml) (Fig. 5F and Table 5). In this case the R_{max} was 0.43 ± 0.01 for C1Bδ (but note that membrane localization was observed in 53% of the cells) and 0.37 ± 0.03 for C1Bθ (membrane localization in 48% of the cells). No membrane translocation was observed for these two domains when 20 μg/ml of DOcPA and 4 μg/ml of DOcG were added together (Table 5). However, when the addition was 20 μg/ml of DOcPA plus 12 μg/ml of DOcG, i.e. high concentrations of both activators, some translocation was observed with R_{max} being 0.44 ± 0.01 for C1Bδ and 0.47 ± 0.01 for C1Bθ (Table 5 and Fig. 5G).

These results confirm that C1Bδ and C1Bθ need higher concentrations of activators for their membrane translocation. Furthermore, C1Bε and C1Bη may respond to DOcPA without the need for DOcG and that a combination of low concentrations of both activators triggers a bigger membrane translocation of these two domains. In contrast, in the case of C1Bδ and C1Bθ they do not respond to DOcPA and high combined concentrations of DOcG (12 μg/ml) and DOcPA (20 μg/ml) are required for their membrane translocations.

4. Discussion

Structural studies have established that all C1 domains have a similar fold [27–33] based on a compact α/β structural unit, which tightly binds two zinc ions. This fold contains an *unzipped* β-sheet that forms a single ligand-binding site for diacylglycerol or phorbol esters (Thr12, Gly23 and Leu21) in its top, surrounded by hydrophobic residues that are involved in membrane insertion [29].

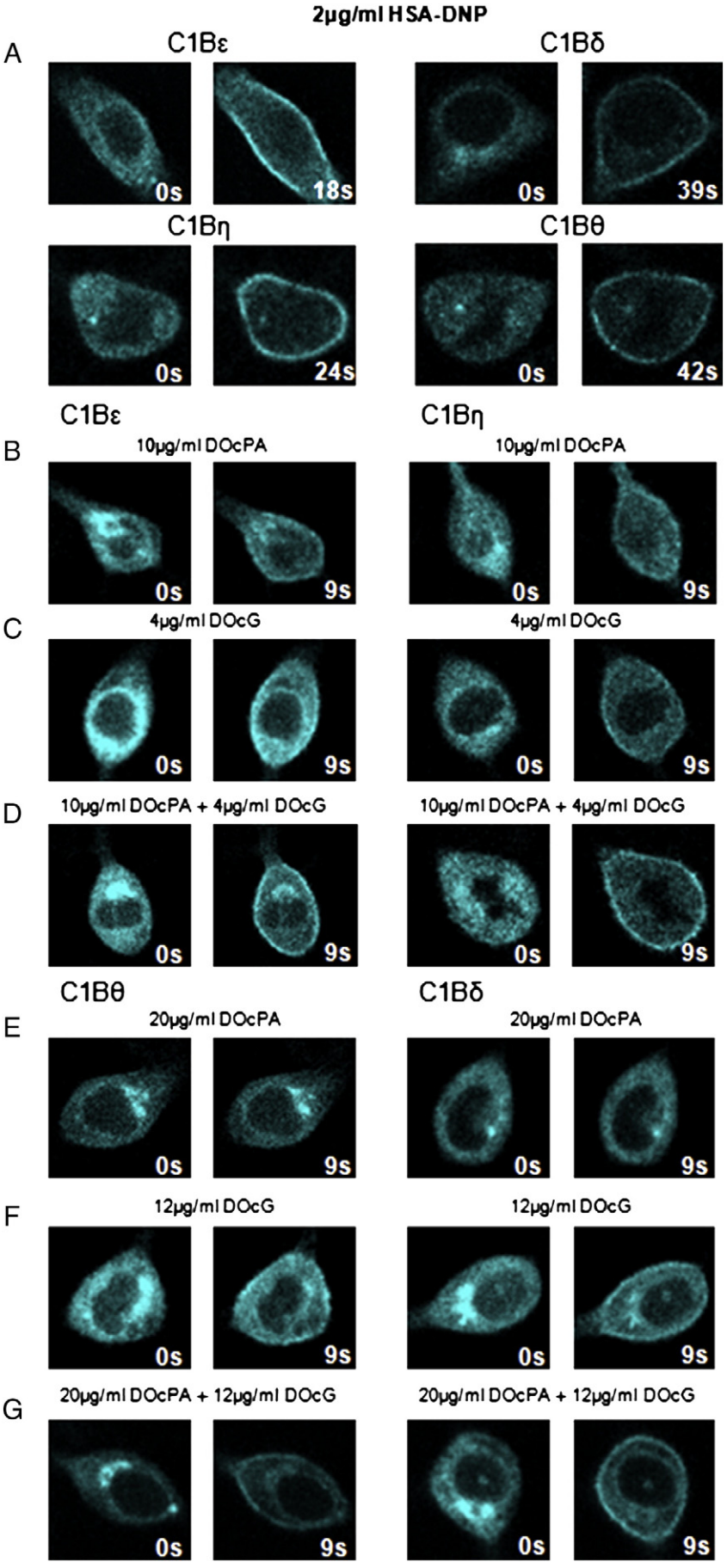
Conventional and novel PKCs contain two C1 domains, C1A and C1B, although the exact function of each of these domains and the reason for the existence of this double domain are not fully clear. Much research effort has focused on trying to understand the specific function of these domains, which we have reviewed in detail elsewhere [2].

Studying C1A domains is hampered by the difficulty involved in obtaining them in soluble form from transfected cell cultures. C1B, however, can be obtained in soluble form and in sufficient amounts to carry out studies like the one reported here, whose objective was to compare the membrane binding affinity of the C1B domains of the four novel PKCs, studying them side by side and using both model membranes and live cells.

As regard the C1B domain, we compared the sequence homology of the four domains from novel PKCs, using the program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the sequences for the C1B domains deduced from Uniprot (http://www.uniprot.org/) for PKCε (code P16054), PKCη (code P23298), PKCδ (code P28867) and PKCθ (Q02111), all of them from mouse. It was seen that whereas ε and η have 84% homology between them, both of them only have 62% homology with δ and 66% with θ. On the other hand, δ and θ present 80% homology.

In the first part we studied the binding of the C1B domains to membranes using a FRET assay. From the binding affinity results (the reciprocal of the apparent K_D values) for the four domains, it can be concluded that C1Bε and C1Bη have a much higher binding affinity than C1Bδ and C1Bθ. This pattern of two well differentiated groups with respect to C1B domains from novel PKCs was confirmed by studies both in the presence and absence of diacylglycerol, when all of them showed similar membrane affinity in the presence of saturating PMA

Fig. 5. Membrane translocation of C1B domains to the plasma membrane of RBL-2H3 cells. Cells were transfected with ECFP-C1Bδ, ECFP-C1Bθ, ECFP-C1Bε and ECFP-C1Bη contructs, as indicated. The pictures show the effect of the addition of antigen (DNP-HSA) or the indicated amounts of soluble diacylglycerol (DOcG) and/or soluble phosphatidic acid (DOcPA).


2μg/ml HSA-DNP
A
C1Bε
C1Bδ
0s
18s
0s
39s
C1Bη
C1Bθ
0s
24s
0s
42s
B
C1Bε
C1Bη
10μg/ml DOcPA
10μg/ml DOcPA
0s
9s
0s
9s
C
4μg/ml DOcG
4μg/ml DOcG
0s
9s
0s
9s
D
10μg/ml DOcPA + 4μg/ml DOcG
10μg/ml DOcPA + 4μg/ml DOcG
0s
9s
0s
9s
E
C1Bθ
C1Bδ
20μg/ml DOcPA
20μg/ml DOcPA
0s
9s
0s
9s
F
12μg/ml DOcG
12μg/ml DOcG
0s
9s
0s
9s
G
20μg/ml DOcPA + 12μg/ml DOcG
20μg/ml DOcPA + 12μg/ml DOcG
0s
9s
0s
9s

concentration (1 mol% of the total lipid). The two groups were also clearly distinguished when the experiments were conducted using living cells and stimulating membrane binding with either an antigen or permable forms of phosphatidic acid and/or diacylglycerol. Note that this division of novel C1B domains into two groups correlates well with the sequence homology mentioned above.

Several observations deserve comment. One is related with the specificity for the different anionic phospholipids studied. C1Bε, C1Bη and C1Bθ were seen to have a higher specificity for POPA, judging from the K_D values. Only C1Bδ had a higher specificity for POPS. If the concentration of anionic phospholipids in the membrane was increased from 5 to 40 mol%, the membrane binding affinity increased by up to one order of magnitude, illustrating the importance of anionic phospholipids for enabling the binding of C1B domains to the membrane. This was also shown by the experiments in which DOG was omitted from the model membranes, especially in the case of C1Bε and C1Bη in the presence of POPA, when the effect of DOG was very modest (in the case of C1Bη the K_D fell from 1.82 ± 0.50 μM in the absence to 1.11 ± 0.03 in the presence of 5 mol% DOG). However, in the presence of PMA at 1 mol%, which is a very high concentration, the membrane binding differences between domains and their specificity for phospholipids, were minimized.

It should be remarked that the influence of the interaction with anionic phospholipids on the membrane binding of C1B domains of novel PKCs was clearly demonstrated by the experiments in the absence of DOG, since in this type of experiment it was very clear that C1Bε and C1Bη had a much higher membrane binding affinity than C1Bδ and C1Bθ, as was the case for all the anionic phospholipids tested. It is to be expected that this would also be the case in physiological conditions for translocation to the cell plasmatic membrane. As regard diacylglycerol, the physiological levels of this lipid in biomembranes were reviewed in [34]. For example, quantitative measurements of diacylglycerol present in stimulated cells have shown that it may reach 1.45 mol% of the total lipid concentration [35] or about 2 mol% [36]. Higher levels have been described in other systems, e.g. the 10 mol% observed in transformed 3T3 cells [37] while in other membranes the levels may vary considerably. In addition to that, high levels may be locally expected in points close to where the enzymes catalyze their formation. 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 [38] or even as high as 11.5 mol% with respect to total lipid [39] or 19 mol% [40] or 25 mol% [41]. With respect to the physiological concentration of phosphatidylserine in the inner monolayer of eukaryotic plasma membranes, such as in erythrocyte or platelet cells, this reaches roughly 20 mol% [42–44], so that the concentration used in this work is within the physiological range.

It is also interesting that PKCη showed a higher membrane binding affinity than PKCε in the absence of DOG and when POPS and POPG were the anionic phospholipids present. Nevertheless, POPA was the anionic phospholipid for which the lowest K_D was observed. Phosphatidic acid is produced in membranes by enzymatic activity through two routes, the action of phospholipase D and that of diacylglycerol kinases. Phosphatidic acid constitutes approximately 1–5% of total cellular lipids [45,46] and is found both in plasma and inner membranes like the Golgi network [47–49]. Nevertheless, it should be taken into account that local concentrations will be considerably higher in the immediate vicinity of the points where phosphatidic acid is being synthesized. It is interesting that phosphatidic acid has been seen to be important in the activation of PKCε [10] and, although it has been shown that this phospholipid binds to the C2 domain of PKCε [10,11,50,51], the findings described here suggest that lipid microdomains containing both PA and diacylglycerol also play an important role in inducing the anchorage of C1 domain in membranes.

The experiments carried out with RBL-2H3 cells confirmed that two groups can be defined within the C1Bs domains of novel PKCs. This was observed when cells were stimulated with DNP-HSA, although differences were found in the time of translocation rather than in the maximum translocation rate. It seems that high concentrations of activators are generated when using this concentration of antigen and thus this experiment has a similarity with the one in which we used PMA (Table 3).

When using permeable activators, it was found that whereas high percentages of C1Bη and C1Bε may be translocated to the cell membrane following the addition of phosphatidic acid, C1Bδ and C1Bθ need the addition of diacylglycerol before their translocation. According to our observations made in model membranes, if enough phosphatidic acid can be incorporated in the cell membranes the four domains will be translocated, but when soluble phosphatidic acid is added only a limited concentration is expected to be reached in the cell membrane, which will not be sufficient for the translocation of C1Bδ and C1Bθ in the absence of diacylglycerol. It was described that direct phosphatidic acid stimulation produces a similar pattern of plasma membrane translocation to that produced by antigen stimulation in RBL-2H3 cells [10]. Previous in vitro experiments in our laboratory demonstrated the preference of the isolated C2 domain of PKC to preferentially bind

Table 5
Plasma membrane translocation parameters in RBL-2H3 cells stimulated with DOcPA and/or DOcG.

Stimulation	*N* cells	M.L., %	R_{max}	$t_{½}$, *s*
C1Bε-ECFP				
20 μg/ml DOcPA	22	96	0.44 ± 0.02	3 ± 1
15 μg/ml DOcPA	31	83	0.40 ± 0.02	3 ± 1
10 μg/ml DOcPA	24	79	0.33 ± 0.03	3 ± 1
5 μg/ml DOcPA	28	46	0.25 ± 0.03	6 ± 1
2 μg/ml DOcPA		0		
12 μg/ml DOcG	29	93	0.46 ± 0.02	3 ± 1
8 μg/ml DOcG	23	78	0.39 ± 0.01	3 ± 1
4 μg/ml DOcG	46	34	0.29 ± 0.03	4 ± 1
2 μg/ml DOcG				
10 μg/ml DOcPA + 4 μg/ml DOcG	21	95	0.51 ± 0.01	3 ± 1
5 μg/ml DOcPA + 4 μg/ml DOcG	18	94	0.41 ± 0.03	3 ± 1
2 μg/ml DOcPA + 2 μg/ml DOcG		0		
C1Bη-ECFP				
20 μg/ml DOcPA	24	96	0.41 ± 0.02	3 ± 1
15 μg/ml DOcPA	22	90	0.37 ± 0.01	3 ± 1
10 μg/ml DOcPA	22	59	0.21 ± 0.02	3 ± 1
5 μg/ml DOcPA		0		
12 μg/ml DOcG	27	92	0.41 ± 0.01	4 ± 1
8 μg/ml DOcG	22	36	0.43 ± 0.01	3 ± 1
4 μg/ml DOcG	31	19	0.26 ± 0.01	6 ± 1
2 μg/ml DOcG		0		
10 μg/ml DOcPA + 4 μg/ml DOcG	27	100	0.53 ± 0.03	2 ± 1
5 μg/ml DOcPA + 4 μg/ml DOcG	31	45	0.34 ± 0.01	3 ± 1
2 μg/ml DOcPA + 2 μg/ml DOcG		0		
C1Bδ-ECFP				
50 μg/ml DOcPA		0		
30 μg/ml DOcPA		0		
20 μg/ml DOcPA		0		
12 μg/ml DOcG	21	53	0.43 ± 0.01	8 ± 1
8 μg/ml DOcG	25	44	0.28 ± 0.02	11 ± 3
4 μg/ml DOcG		0		
20 μg/ml DOcPA + 4 μg/ml DOcG		0		
20 μg/ml DOcPA + 12 μg/ml DOcG	32	81	0.44 ± 0.01	8 ± 1
C1Bθ-ECFP				
50 μg/ml DOcPA		0		
30 μg/ml DOcPA		0		
20 μg/ml DOcPA		0		
12 μg/ml DOcG	21	48	0.37 ± 0.03	3 ± 1
8 μg/ml DOcG	21	28	0.29 ± 0.02	4 ± 1
4 μg/ml DOcG		0		
20 μg/ml DOcPA + 4 μg/ml DOcG		0		
20 μg/ml DOcPA + 12 μg/ml DOcG	41	75	0.47 ± 0.01	5 ± 1

M.L. is membrane localization and indicates the percentage of cells responding to DOcPA and/or DOcG stimulation with plasma membrane translocation. R_{max} is the maximal relative increase in plasma membrane localization of the domain. $t_{1/2}$ is the half time of translocation.

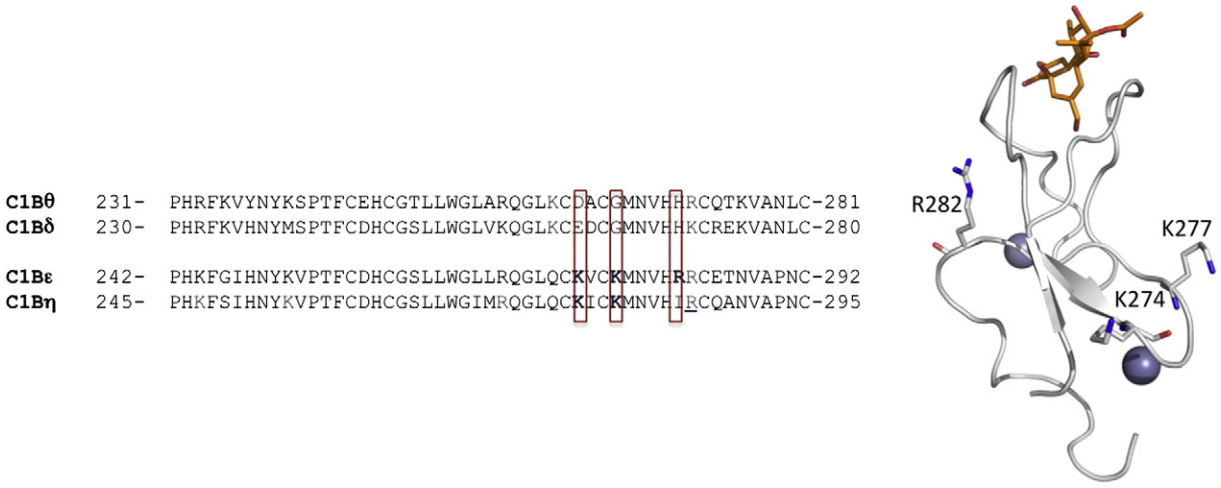


Fig. 6. Alignment of the amino acid sequences of the C1B domains of novel PKCs. The highlighted residues correspond to those positively charged residues (K and R) that are different between the couple C1Bδ–C1Bθ and the couple C1Bε–C1Bη. Residue R286 of C1Bη is underlined because is close to I285 that occupies a position analog to R282 in C1Bε, this is being one of the apparently significant differences with respect to couple C1Bδ–C1Bθ. The molecular model shown corresponds to C1Bε, modeled on the basis of C1Bδ (1PTR) as described in the text. Zn^{2+} cations are shown as blue spheres and phorbol ester in orange.

phospholipid vesicles containing phosphatidic acid [52,53]. The results obtained previously also suggest that the full-length enzyme needs a continuous source of PtdOH to translocate to the plasma membrane in physiological conditions [10]. We have also used in this paper exogenous PtdOH (DiC8-PtdOH) because it can be added to the cell culture medium and is incorporated rapidly into cell membranes where it subsequently participates in cellular functions [54].

It is known that diacylglycerol may be formed from phosphatidic acid by the action of phosphatases in cells. Therefore it may be suspected that the exogeneous PA that is added in some experiments reported above may be transformed in DAG, but available experimental evidence indicates that the formation of both DAG and PA upon cell stimulation occurs with $t_{1/2}$ of several minutes [55,56]. However we observed that the translocation to membrane of C1 domains induced by exogeneous PA has $t_{1/2}$ of a few seconds. Furthermore, it is reported above (Fig. 5) that C1Bδ and C1Bθ were not translocated to membranes even after 10 min of the addition of 50 mg /ml of soluble phosphatidic acid, strongly indicating that the effect is really due to phosphatidic acid and not to the formation of diacylglycerol by hydrolysis of this exogeneously added phospholipid.

We have observed that C1Bε and C1Bη are translocated more rapidly to the membrane than C1Bδ and C1Bθ and hence this may be due to both activators acting together. However C1Bδ and C1Bθ need a longer timer and this may be due to the fact that they require a higher concentration of both activating lipids. In order to identify the role played by each of these activating lipids we used soluble forms added exogenously. The results showed that the addition of soluble PA only triggered the translocation of C1Bε and C1Bη in a very rapid way (in a seconds time scale) whereas the translocation of C1Bδ and C1Bθ were not triggered.

It would be interesting to ascertain whether ~~if~~ the differences observed between the C1Bs of novel PKCs are related with their physiological specializations. It has been described, for example, that the activation of PKCε and PKCδ has opposing consequences in ischemic myocardium [57]. Whereas PKCε activation has a cardioprotective effect [58], PKCδ activation mediates much of the acute injury induced after transient myocardial ischemia [59,60]; also PKCθ and PKCη play different roles in signal transductions in T cells. When an immunological synapse is formed between a T cell and an antigen-presenting cell, PKCθ concentrated in the central region of the synapse, while PKCη diffuses over the whole area of the synapse, suggesting that each may bind to a different activator in the membrane [61]. In addition, the deficiency of PKCη or PKCθ in T cells had opposing effects in knockout mice, since PKCη knockout had a higher CD4 to CD8 ratio [61]. Given these differences in functions and effects within the same cells, it seems plausible that PKCε and PKCη may have different degrees of sensitivity to activators from PKCδ and PKCθ (as we have shown in this paper): each of these isoenzymes would be translocated to the area of the plasma membrane or to the organelle where there is a more favorable combination of diacylglycerol and anionic phospholipid, even though some of them may be more responsive to low concentrations of one of these activators.

It is widely known that the C1 domain binds to the membrane by interacting with DAGs (or phorbol esters). It has been observed that each C1 domain may have preference for a certain subcellular membrane and these differences help to explain the divergent localization and distinct functional roles of the full-length proteins, which contains them. This selectivity has been explained by their capacity to discriminate between DAG pools [62]. But here we may demonstrate that electrostatic interactions provided by anionic phospholipids also make an important contribution to the specific targeting of C1 domains to cell membranes. These protein–phospholipid interactions have been found to be quite important for the binding of many extrinsic proteins, such as cytochrome *c* [63,64], myelin basic protein [65], phospholipases [66], K-Ras [67], charybdotoxin [68], cardiotoxin [69] and Aβ peptides [70].

It should be mentioned that diacylglycerols bearing short fatty acyl chains show a very high activation capacity with respect to PKC [38]. Both phosphatidic acid [71] and diacylglycerol [34], are lipids that may induce negative curvature and this may favor protein insertion in the membrane but this effect will be the same for all the isoenzymes and therefore will not affect the conclusions obtained about the different behavior of the isoenzymes with respect to membrane binding.

In order to examine the possible structural basis for the observed differences between the two couples of C1B domains of novel PKCs we have elaborated an alignment of their sequences as shown in Fig. 6. We highlight these positively charged residues that are not conserved when comparing both couples of domains and that are present in C1Bε and C1Bη, such as K274, K277 and R282 (C1Bε) and K277 and K280 (C1Bη). In the case of C1Bη, the residue aligned with R282 in C1Be is however I285 but it might be that the following residue (R286) may compensate for that. Site-targeting mutations should be carried out to confirm these suggestions.

Fig. 6 also shows a molecular modeling of C1Bε, although a high resolution structure for this domain is not known. To overcome this we have carried out a modeling by using C1Bδ as a basis (PDB 1PTR) and using Geno3D website (Pôle Bioinformatique Lyonnais Geno3D, http://pbil.ibcp.fr/htm/index.php). The model includes a molecule of

phorbol ester occupying the cavity as was observed previously [29]. Since the phorbol ester molecule must be embedded in the membrane, the residues highlighted in the alignment shown in the model are most probably located at the membrane lipid–water interface and this is compatible with their possible interaction with the negatively charge phospholipids of the membrane.

Taken together, our results show that (i) C1B domains from novel PKCs have different binding affinities for model membranes depending on the presence or not of diacylglycerol and of specific anionic phospholipids, (ii) this is also observed in RBL-2H3 cells in which the different C1B domains were expressed (iii) two groups of C1B domains from novel PKCs can be established as a function of their membrane binding affinity: those from PKCε and PKCη with a higher membrane binding affinity and those with PKCδ and PKCθ with a low affinity.

Acknowledgement

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