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The Simultaneous Production of Phosphatidic Acid and Diacylglycerol Is Essential for the Translocation of Protein Kinase Cε to the Plasma Membrane in RBL-2H3 Cells

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To evaluate the role of the C2 domain in protein kinase Cε (PKCε) localization and activation after stimulation of the IgE receptor in RBL-2H3 cells, we used a series of mutants located in the phospholipid binding region of the enzyme. The results obtained suggest that the interaction of the C2 domain with the phospholipids in the plasma membrane is essential for anchoring the enzyme in this cellular compartment. Furthermore, the use of specific inhibitors of the different pathways that generate both diacylglycerol and phosphatidic acid has shown that the phosphatidic acid generated via phospholipase D (PLD)-dependent pathway, in addition to the diacylglycerol generated via phosphoinosite-phospholipase C (PLC), are involved in the localization of PKCε in the plasma membrane. Direct stimulation of RBL-2H3 cells with very low concentrations of permeable phosphatidic acid and diacylglycerol exerted a synergistic effect on the plasma membrane localization of PKCε. Moreover, the in vitro kinase assays showed that both phosphatidic acid and diacylglycerol are essential for enzyme activation. Together, these results demonstrate that phosphatidic acid is an important and essential activator of PKCε through the C2 domain and locate this isoenzyme in a new scenario where it acts as a downstream target of PLD.

INTRODUCTION

The protein kinase Cε (PKCε) isoenzyme, which belongs to the group of novel PKCs, has been linked with the regulation of several biological processes, including neuronal differentiation (Brodie *et al*., 1999; Zeidman *et al*., 1999), antiviral resistance (Pfeffer *et al*., 1991), hormone secretion (Akita *et al*., 1994), transporter regulation (Lehel *et al*., 1994; Liedtke *et al*., 2002), and integrin-dependent signaling (Ivaska *et al*., 2002, 2003). Structural studies have shown that the C2 domain of PKCε exhibits two areas at the top surface of the molecule that might participate in its binding to anionic membranes (Ochoa *et al*., 2001). In vitro experiments have confirmed the ability of both C2 domain and full-length PKCε to bind to negatively charged phospholipid vesicles in a Ca^{2+}-independent manner, similarly to that seen for other novel PKC isoenzymes (Medkova and Cho, 1998; Garcia-Garcia *et al*., 2001; Ochoa *et al*., 2001). However, the exact mechanism by which the enzyme anchors in the plasma membrane is still unknown.

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Abbreviations used: DAG, diacylglycerol; DGK, diacylglycerol kinase; DiC8, 1,2-dioctanoylglycerol; DNP-HSA, dinitrophenyl-human serum albumin; PC-PLC, phosphatidylcholine-phospholipase C; PI-PLC, phosphoinosite-phospholipase C; PIP5, phosphatidylinositol 4-phosphate 5-kinase; PLD, phospholipase D; PtdOH, phosphatidic acid; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate.

The C2 domain of PKCε has been involved in several protein–protein interactions. For example, it has been demonstrated that the coatomer protein β′-COP (also called RACK2) interacts with PKCε (Csukai *et al*., 1997). Recent findings have shown that PKCε can also interact with RACK1, positively regulating the integrin-dependent adhesion, spread, and motility of human glioma cells (Besson *et al*., 2002). Another function attributed to this interaction with RACK1 is that it serves as a scaffold to anchor the enzyme close to the cystic fibrosis transmembrane regulator (Liedtke *et al*., 2002). Nevertheless, besides protein–protein interactions, no other roles have been attributed to the C2 domain of PKCε in biological systems and the mechanisms operating in the phospholipid-dependent translocation of this enzyme are still not well characterized in vivo, leaving many questions still unresolved. For example, it is unclear to what extent negatively charged phospholipids are involved in the plasma membrane translocation of the enzyme and what exactly is the molecular mechanism driving this enzyme–membrane interaction after physiological activation of the membrane receptors.

The cross-linking of the high-affinity IgE receptor (FcεRI) with antigen in mast or basophilic cells stimulates a number of lipid-signaling events, which include the activation of phospholipase Cγ, phospho-inositide 3-kinase, and phospholipase D (PLD) (Schneider *et al*., 1992; Brown *et al*., 1998; Djouder *et al*., 2001) and many serine/threonine kinases (Millard *et al*., 1989; Park *et al*., 1991; Gruchalla *et al*., 1990), including the PKC family among others (Ozawa *et al*., 1993). However, it is not well established how PLC and PLD, or other enzymes, participate in the generation of the different activators of the variety

Table 1. Percentage of β-hexosaminidase release in RBL-2H3 cells in response to DNP-HSA

No pretreatment	U73122	1-Butanol	R59022	D609	Propranolol
25.41 ± 1.68	3.3 ± 0.63	3.51 ± 0.45	20.06 ± 0.6	25.7 ± 1.29	2.2 ± 1.5

RBL-2H3 cells were primed with 0.5 μg/ml anti-IgE antibody for 16 h and then exposed for 10 min to each inhibitor before stimulation with 4 μg/ml DNP-HSA. The data are the mean of four different experiments ± SD.

of PKCs or how they contribute to the localization and activation of each isoenzyme.

RBL-2H3 cells (a rat basophilic cell line) contain at least five species of PKC isoenzymes: α, β, δ, ϵ, and ζ. More particularly, it has been suggested that PKCϵ serves as a link between the aggregation of the IgE receptor and the subsequent expression of *c-fos* and *c-jun* (Razin *et al*., 1994). In addition, it has been demonstrated that upon antigen stimulation, this isoenzyme translocates to the plasma membrane where it contributes to cell proliferation (Chang *et al*., 1997).

In the present study, we have examined the effect of the substitution of several amino acidic residues localized in the C2 domain of PKCϵ on the localization of the enzyme in the plasma membrane, after activation of FcϵRI. The results have shown that loops 1 and 3 of the C2 domain participate in the membrane anchorage of the enzyme by interacting with phosphatidic acid (PtdOH). This phospholipid is mainly generated via PLD, although a small contribution on the part of diacylglycerol kinase (DGK) cannot be ruled out. Additionally, an essential interaction with diacylglycerol (DAG), which is generated by phosphoinosite (PI)-PLCs, is necessary to firmly anchor the enzyme in the plasma membrane and to activate its catalytic activity. In conclusion, PKCϵ has been defined as a new target for biologically generated PtdOH and placed in a new context as a downstream target of PLD.

MATERIALS AND METHODS

Construction of Expression Plasmids

PKCϵ cDNA was a gift from Drs. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). N-Terminal fusion of rat PKCϵ to green fluorescent protein (GFP) was generated by inserting cDNA into *Bgl*II and *Bam*HI sites of the plasmid enhanced green fluorescent protein (pEGFP)-N3 (BD Biosciences Clontech, Palo Alto, CA) mammalian expression vector. The C2 domain mutants were generated by polymerase chain reaction by using the modified C2 domains generated in our previous work as a template (Ochoa *et al*., 2001) before being introduced into the *Bgl*II and *Bcl*I sites of PKCϵ-GFP. The primers used were 5′GCCAGATCTATGGTAGTGTTCAATG and 3′GAAAAGTCTACGTGATCATCGATC. Then, both wild-type and mutant genes were subcloned into the *Xba*I and *Kpn*I sites of the pCGN vector (Tanaka and Herr, 1990), thus allowing the expression of PKCϵ fused 3′ to the hemagglutinin (HA) epitope. All constructs were confirmed by DNA sequencing.

Previous studies have shown that a C-terminal GFP tag does not affect the localization, catalytic activity or the cofactor dependence of PKCϵ (Shirai *et al*., 1998). Additionally, the stability and viability of the mutated proteins were studied by using specific activity measurements as shown below.

Cell Culture and Transfections

Human embryonic kidney (HEK)293 cells were grown in DMEM with 10% fetal calf serum and transfected following the calcium phosphate method described by Wigler *et al*. (1977). 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 Bolsover *et al*., (2001). 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. Cells were electroporated in a GenePulser (Bio-Rad, Hercules, CA) with two 500-V pulses. The cells were 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). 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 (DiC8) and PtdOH (DiC8-PtdOH) 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 >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.

When used, the various phospholipase inhibitors were present 10 min before stimulation (4 μg/ml dinitrophenyl-human serum albumin [DNP-HAS]) at the following concentrations: 30 μM U73122, 30 μM D-609, 50 mM 1-butanol, 100 μM propranolol, and 20 μM DGK inhibitor II (R59022). To demonstrate that the inhibitors we used were selective under the conditions of these experiments, we examined their effect on β-hexosaminidase release in RBL-2H3 cells transfected with PKCϵ-GFP. The results are presented in Table 1. As expected, IgE-dependent release was inhibited by U73122, 1-butanol, and propranolol (Schneider *et al*., 1992; Cissel *et al*., 1998). A slight decrease in the release of β-hexosaminidase was observed when DGK inhibitor was used, and no effect on secretion was detected when the cells were preincubated with D-609 (Table 1). The β-hexosaminidase release was measured as described by Ozawa *et al*. (1993).

When possible, additional control experiments were performed with U73343, which is an inactive analogue of U73122. 1-Butanol can replace water in the reaction catalyzed by PLD to produce choline and phosphatidylbutan-1-ol (PBut) instead of phosphatidic acid and PtdOH; however, 2-butanol does not compete in this reaction and thus can be used as an appropriate control for the inhibition of the PA generated by PLD.

Confocal Microscopy

Cells expressing various PKCϵ-EGFP 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 488 nm and emission wavelengths at 500–525 nm for GFP. During imaging, cells were stimulated with antigen (DNP-HAS; Sigma-Aldrich Quimica, S.A.) or other agents as described. Series of 60–120 confocal images were recorded for each experiment at time intervals of 5 s.

Image Analysis

Background was subtracted from 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 (Meyer and Oancea, 2000). The relative increase in the amount of enzyme localized in the plasma membrane for each time point was calculated by using the ratio $R = (I_{mb} - I_{cyt})/I_{cyt}$ where I_{mb} is the fluorescence intensity at the plasma membrane and I_{cyt} is the average cytosolic fluorescence intensity. Mean values are given ± SE of the mean.

Purification of Protein Kinase Cϵ and Its Mutants

HEK293 cells (9-cm plates) were transfected with 10 μg of cDNA of pCGN-PKCϵ and the different mutants. Cells were harvested at 48 h postransfection, pelleted, and resuspended in lysis buffer (5 ml of buffer/g cells) containing 20 mM Tris pH 8, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptine, 100 μM Na_3VO_4, 50 mM NaF, and 20 mM *n*-octyl-β-D-glucopyranoside. The pellet was disrupted by sonication and the resulting lysate was centrifugated at 12,500 rpm for 30 min at 4°C. The supernatant was applied to a DEAE-Sephacel column and equilibrated with E buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EGTA, 0.5 mM EDTA,

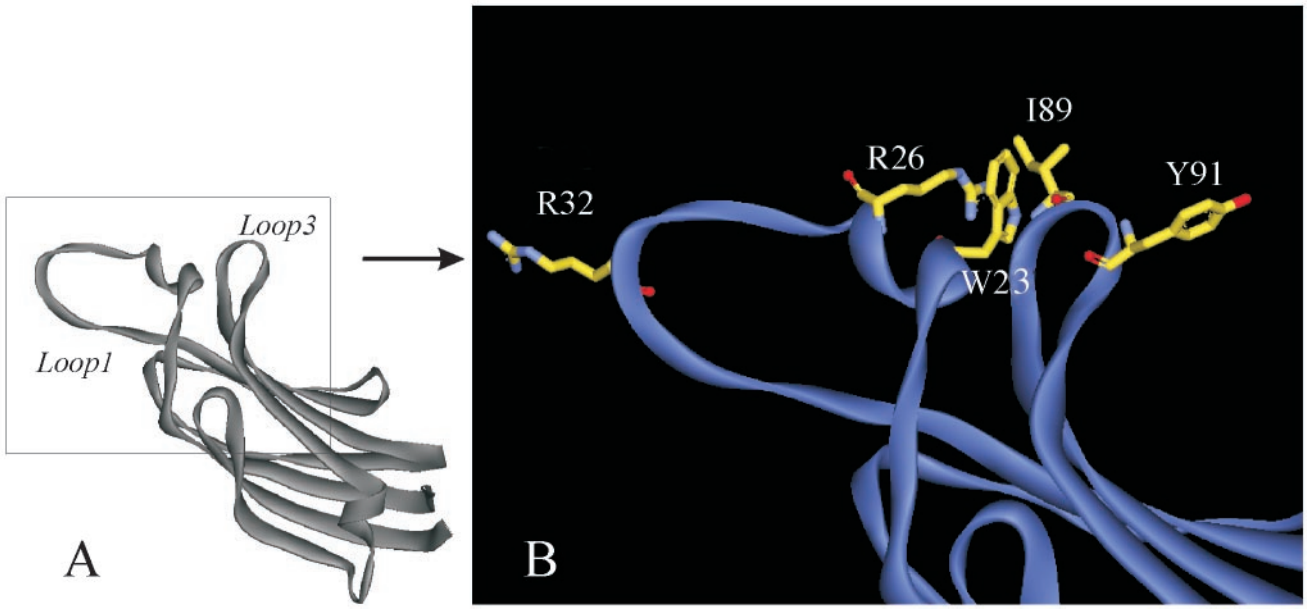

Figure 1. (A) Overall structure of the C2 domain of PKCε. The central structural feature of this C2 domain is an eight-stranded antiparallel β-sandwich of type II topology. Loop 1 corresponds to the connection between β1 and β2 strands and, loop 3 corresponds to the connection between β5 and β6 strands. The square box is amplified in B. (B) Represents the top region of the C2 domain, corresponding to loops 1, 2, and 3. The side chains of the mutated amino acids are shown in yellow. The three mutants generated in this work are: PKCε-W23A/R26A/R32A in loop 1, PKCε-I89N and PKCε-Y91A in loop 3.

and 10 mM β-mercaptoethanol). The bound proteins were eluted from the column by the application of a salt gradient (0–1 M NaCl in buffer E) at a flow rate of 0.5 ml/min. Protein was concentrated by using a 30K Ultrafree centrifugal filter device (Millipore, Billerica, MA). The protein was then aliquoted and stored at −80°C in the presence of 10% (vol/vol) glycerol and 0.05% (vol/vol) Triton X-100.

Preparation of Large Unilamellar Vesicles

The lipid mixtures were generated by mixing chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glicero-3-phosphocoline (POPC), 1-palmitoyl-2-oleoyl-*sn*-glicero-3-phosphate (POPA), and 1,2-*sn*-dioleylglycerol (DOG) (Avanti Polar Lipids, Alabaster, AL) and dried from the organic solvent under a stream of nitrogen and then further dried under vacuum for 90 min. Sucrose-loaded vesicles were prepared as described by Rebecchi *et al*., (1992).

Kinase Activity Assay

The kinase activity was assayed in vitro with purified wild-type and mutants PKCε by measuring the incorporation of ^{32}Pi into the substrate histone III-S. The reaction was started by addition of 0.033 μg of PKCε to a 48-μl reaction mixture containing 20 mM Tris-HCl pH 8.0, 0.2 mM EGTA, 5 mM MgCl, 0.2 mg/ml histone III-S, 0.05 mM ATP with [^{32}P]ATP (200,000 cpm/nmol) and 0.2 mM final concentration of lipids. After 20 min, the reaction was stopped using 1 ml of 25% trichloroacetic acid and 1 ml of 0.05% bovine serum albumin. The reaction mixture was then stored on ice for 30 min and the proteins were colleted on a 2.5-cm-diameter glass fiber filter and washed twice with 10% trichloroacetic acid. The incorporation of ^{32}Pi was measured by liquid scintillation counting. Background specific activity in the absence of lipids was also measured and subtracted to that obtained in the presence of the distinct phospholipid mixtures. Additional control experiments were performed with mock cell lysates to eliminate the endogenous activity, which represented <1% of the total enzyme activity measured.

RESULTS

PKCε Translocates to the Plasma Membrane of RBL-2H3 Cells after the Activation of the IgE Receptor

Previous crystallographic work suggested that amino acidic residues located in loops 1 and 3 of the C2 domain of PKCε participated in the interaction of the domain with liposomes containing negatively charged phospholipids (Ochoa *et al*., 2001). To study the role of these residues in the physiological membrane targeting of the enzyme, we generated three constructs by site-directed mutagenesis containing the following substitutions: W23A/R26A/R32A, located in loop 1 (PKCε-W23A/R26A/R32A-GFP), and I89N (PKCε-I89N-GFP) and Y91A (PKCε-Y91A-GFP) both located in loop 3 (Figure 1, A and B). A triple substitution was chosen in the case of loop 1, because previous in vitro binding assays showed that a single substitution of these residues does not affect the membrane binding properties of the domain (Ochoa *et al*., 2001). Additionally, both wild-type and mutant proteins were fused to the amino-terminal of green fluorescent protein (PKCε-GFP), which enabled us to study the spatio-temporal localization of the enzyme by using confocal microscopy in time-lapse experiments.

Figure 2A shows images of IgE-stimulated RBL-2H3 cells expressing PKCε-GFP. In unstimulated cells, the protein was uniformly distributed through the cytosol (Figure 2A, a), whereas stimulation with 4 μg/ml DNP-HSA resulted in a translocation of the enzyme to the plasma membrane (Figure 2A, b). When the relative increase in plasma membrane over cytosolic fluorescence intensity (R) was calculated at each time point, it was observed that maximal translocation was reached after 105 s of antigen stimulation (Figure 2B), indicating the slow progress of plasma membrane localization (1.75 min) especially compared with the classical isoenzymes (26 s) (Oancea and Meyer, 1998; Bolsover *et al*., 2003). The protein was anchored to the plasma membrane for 108 s and started to revert slowly to the cytosol, reaching half plasma membrane dissociation 455 s after antigen stimulation.

To test whether a membrane-permeant diacylglycerol (DiC8) was able to bring about the localization of the enzyme in the plasma membrane, the cells were stimulated first with 4 μg/ml DNP-HSA. When the protein was half-dissociated from the

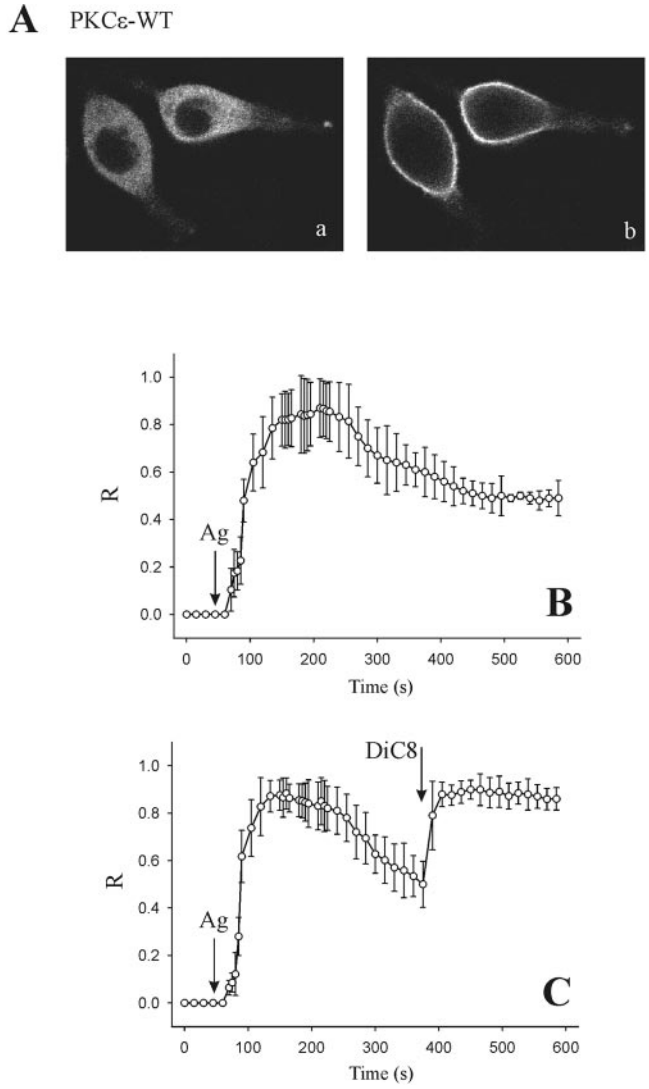

Figure 2. (A) Receptor-induced translocation of PKCϵ to the plasma membrane. The FcϵRI of RBL-2H3 cells were sensitized by the addition of 0.5 μg/ml anti-DNP IgE for 16 h. Confocal images were obtained in a time lapse experiment, during which a series of 120 images were recorded at 5-s intervals. Receptor cross-linking was performed by addition of 4 μg/ml DNP-HSA after 45 s of recording. The images shown were recorded at 0 (a) and 200 s (b). (B) Average data of the time course of plasma membrane translocation of PKCϵ after stimulation of the receptor by antigen. R, relative plasma membrane translocation calculated as explained in MATERIALS AND METHODS. (C) Average data of the time course of plasma membrane translocation of PKCϵ upon antigen and DiC8 stimulation, DiC8 was added after 370 s of recording.

plasma membrane, 25 μg/ml DiC8 was added, maximal enzyme translocation to the plasma membrane being reached in 31 s, whereas the protein remained anchored to the plasma membrane for at least 200 s (Figure 2C).

Role of Lipid-binding Residues Located in the C2 Domain of PKCϵ in the Antigen-dependent Translocation of the Enzyme to the Plasma Membrane

To determine the role of the C2 domain in the antigen-dependent translocation of PKCϵ, we examined the effect of

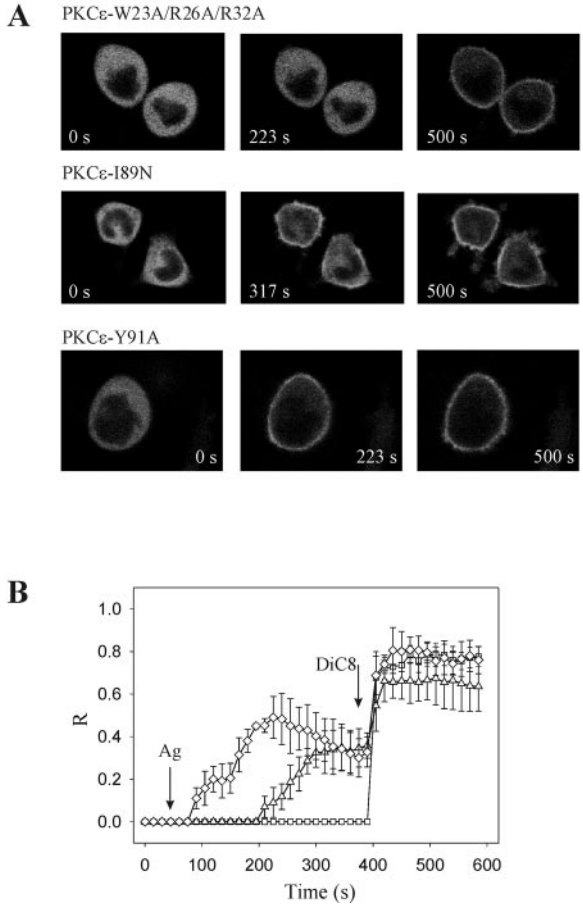

Figure 3. (A) Confocal fluorescence images of RBL-2H3 cells expressing the different phospholipid-binding mutants, PKCϵ-W23A/R26A/R32A, PKCϵ-I89N, and PKCϵ-Y91A at different points of the time-lapse experiment. Antigen was added after 45 s and DiC8 after 395 s of recording. (B) Average of the time courses of plasma membrane localization of PKCϵ-W23A/R26A/R32A (□), PKCϵ-I89N (△) and PKCϵ-Y91A (◇) under the conditions stated above.

antigen stimulation on the plasma membrane translocation of each of the mutant proteins generated. In the case of the PKCϵ-W23A/R26A/R32A-GFP mutant, antigen stimulation was not sufficient to induce the translocation of the protein to the membrane, and only when DiC8 was added to the incubation media did the protein anchor itself to the membrane permanently (Figure 3, A and B). When the cells were transfected with PKCϵ-I89N-GFP mutant, a very low and partial degree of membrane localization was observed (Figure 3A), the maximal translocation ratio (R) calculated being 0.34 ± 0.09 272 s after antigen stimulation (Figure 3B). A less damaging effect was observed when Y91 was substituted by A (Figure 3A), in which case partial translocation (R = 0.49 ± 0.1) was obtained 178 s after antigen stimulation (Figure 3B). Both these two mutants also translocated to the plasma membrane after the addition of DiC8.

These data suggest that both loop 1 and 3 are critical for PKCϵ membrane targeting and might interact with some other activators probably generated upon antigen stimulation besides diacylglycerol. Another possibility is that the C2 domain cooperates with the C1 domain by increasing its affinity for diacylglycerol, the mutagenesis producing an overall decrease in the affinity of the enzyme for this compound.

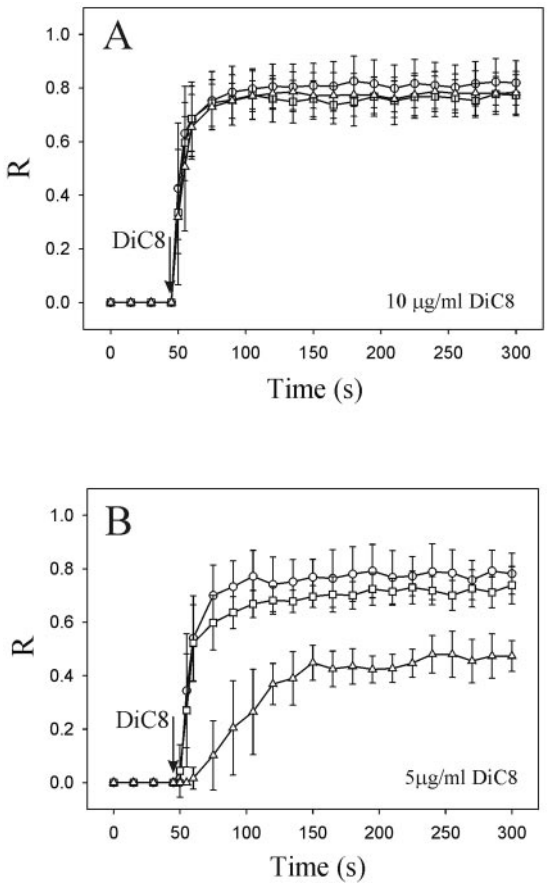

Figure 4. Average of the time courses of plasma membrane localization when RBL-2H3 cells were transfected with wild-type PKCε (○), PKCε-W23A/R26A/R32A (□) and PKCε-I89N (△) and stimulated with 10 μg/ml DiC8 (A) and 5 μg/ml DiC8 (B). R, relative plasma membrane translocation.

Effect of Direct Stimulation with DiC8 on the Membrane Translocation of PKCε and the Different Mutants

To directly determine the role of diacylglycerol in the plasma membrane translocation of PKCε, RBL-2H3 cells were transfected with wild-type protein, PKCε-W23A/R26A/R32A-GFP, or PKCε-I89N-GFP mutants. The cells were then stimulated with different concentrations of DiC8. Figure 4A shows that no differences in localization were found between the wild-type and the two mutants when the cells were stimulated with 10 μg/ml DiC8. Furthermore, the proteins bound to the plasma membrane 27 s after DiC8 addition. Similar results were obtained when the cells were stimulated with 25 μg/ml DiC8 (our unpublished data). However, when the concentration of DiC8 was decreased to 5 μg/ml (Figure 4B), the wild-type protein translocated to the plasma membrane 35 s after stimulation, and the PKCε-W23A/R26A/R32A mutant exhibited a slightly slower translocation profile. Surprisingly, the effect was greater in the PKCε-I89N mutant, which only half-translocated ($R = 0.48 \pm 0.09$) 105 s after DiC8 stimulation (Figure 4B). These experiments suggest that I89 located in loop 3 of the C2 domain might enhance the affinity of the C1 domain for diacylglycerol or even participate directly in the interaction.

It is important to note that the very low degree of inhibition obtained in the plasma membrane localization of the mutants after DiC8 stimulation differed from the dramatic inhibition obtained after stimulation with DNP-HSA, strongly suggesting that the force driving the enzyme translocation to the plasma membrane under physiological conditions is not only DiC8 but also other components generated in this process, such as phosphatidic acid.

Involvement of PLD, DGK, and PI-PLC in the Plasma Membrane Localization of PKCε

To shed light on the plasma membrane localization of PKCε under physiological conditions, we used several specific inhibitors of the different signaling pathways related with the biological generation of both DAG and PtdOH. Thus, we used U73122, a specific inhibitor of PI-PLC (Bleasdale *et al*., 1990), and D-609, which has been reported to inhibit phosphatidylcholine (PC)-PLC (Muller-Decker, 1989), both of which impede DAG synthesis from phosphatidylinositol 4,5-bisphosphate and PC, respectively. One of the main ways in which PtdOH is generated in the cell is by the activation of PLD, which hydrolyzes PC to produce PtdOH and choline. The primary alcohol, 1-butanol, can effectively replace water in this reaction to produce choline and phosphatidylbutan-1-ol, thus inhibiting PtdOH synthesis (Cissel *et al*., 1998). Another way of blocking the synthesis of PtdOH is to use propranolol, which directly binds to PtdOH and to phosphatidic acid phosphohydrolase, inhibiting the synthesis of DAG (Koul and Hauser, 1987). An additional source of PtdOH in cell signaling is DGK, which phosphorylates DAG to produce PtdOH, a reaction we blocked by using DGK inhibitor II (R59022) (de Chaffoy de Courcelles *et al*., 1985). We then investigated the effect of each drug on the antigen-dependent membrane localization of PKCε.

RBL-2H3 cells were transfected with PKCε-GFP and primed with anti-IgE antibody for 16 h. Before antigen was added, they were incubated for 10 min with each inhibitor (Figure 5). In resting cells, the protein was localized in the cytosol in all cases, but when the cells were stimulated with 4 μg/ml DNP-HSA, protein translocation to the plasma membrane was completely inhibited by U73122, 1-butanol, and propranolol (Figure 5, a, b, e, f, g, and h). When possible, control experiments were performed with U73343 and 2-butanol, neither of which competed in these reactions, allowing PKCε to localize in the plasma membrane (our unpublished data). An intermediate effect was observed when DGK inhibitor II was used at saturating concentrations of 20 μM (Figure 5, i and j), probably reflecting a smaller contribution of DGK to the generation of PtdOH in this pathway, but more importantly, showing that several sources of PtdOH are needed for PKCε localization. No inhibitory effect on the membrane localization of PKCε was observed when the cells were incubated with D-609, demonstrating that PC-PLC plays no role in this process.

These data suggest that both DAG generated by PI-PLC and PtdOH generated mainly via PLD are necessary for proper PKCε membrane docking, the absence of either completely impeding the localization of this enzyme.

However, the final product of the reaction initiated by PLD is DAG (Figure 10A), and thus, with the use of 1-butanol and propranolol, the possibility cannot be ruled out that instead of the first product (PtdOH), it is the second product (DAG) of the reaction that is responsible for the PKCε plasma membrane localization. Furthermore, it cannot be discarded that there may be a direct effect of the inhibitors on the diacylglycerol-dependent translocation of the enzyme. To address this question, the DiC8-dependent PKCε localization was studied in the presence of 50 mM 1-butanol or 100 μM propranolol. When the cells were stimulated with 10 μg/ml DiC8, no differences in the plasma membrane localization of PKCε were observed be-

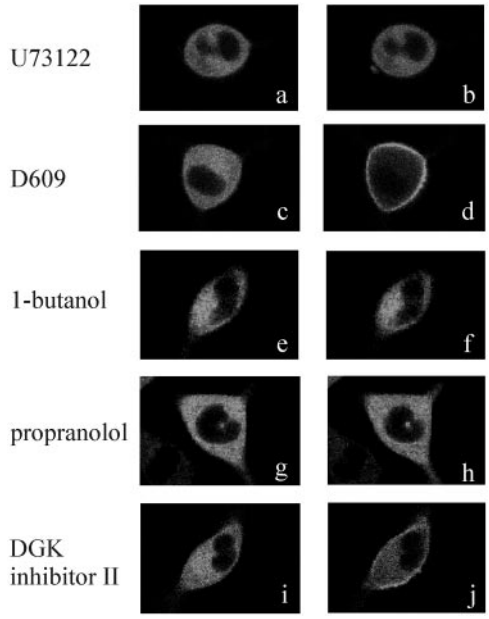

Figure 5. The cultures were preincubated at 37°C with the appropriate concentrations of inhibitors for 10 min before the addition of 4 μg/ml DNP-HSA. a, c, e, g, and i represent the cells before antigen stimulation. b, d, f, h, and j represent the same cells 200 s after antigen addition. The inhibitors were used at 30 μM U73122 (a and b), 30 μM D-609 (c and d), 50 mM 1-butanol (e and f), 100 μM propranolol (g and h), and 20 μM DGK inhibitor II (i and j). The cells shown are representative of four independent assays.

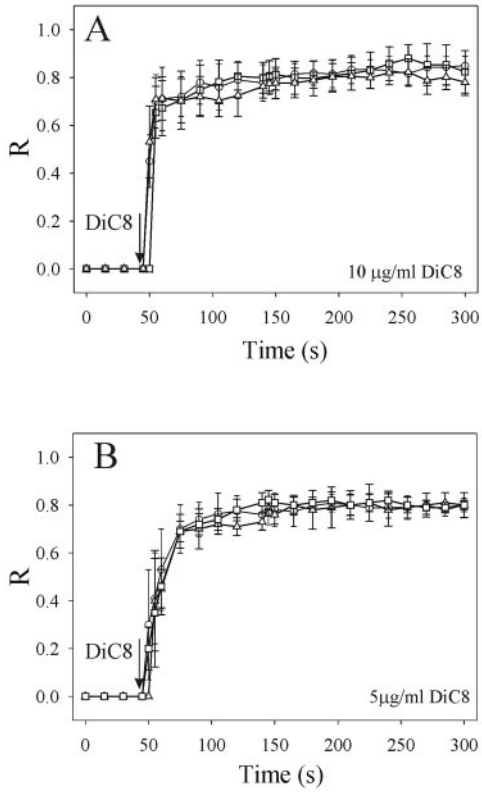

Figure 6. Average of the time courses of plasma membrane localization when RBL-2H3 cells were transfected with wild-type PKCε and stimulated with 10 μg/ml DiC8 (A) and 5 μg/ml DiC8 (B) in the absence (○) and in the presence of 50 mM 1-butanol (□) or 100 μM propranolol (△). R, relative plasma membrane translocation.

tween the cells in the absence and in the presence of each inhibitor (Figure 6A). Similar results were obtained when the cells were stimulated with lower concentrations of DiC8 (5 μg/ml), and neither 1-butanol nor propranolol produced an inhibitory effect on the localization of PKCε (Figure 6B). Together, these data suggest that no additional inhibitory effects over the direct diacylglycerol-dependent translocation of the enzyme seem to occur and most probably, the effect observed when both 1-butanol and propranolol were used as inhibitors in the antigen-dependent localization of PKCε is derived from the direct inhibition of the generation of PtdOH.

Direct Phosphatidic Acid Stimulation Produces a Similar Pattern of Plasma Membrane Translocation to That Produced by Antigen Stimulation

Previous in vitro experiments in our laboratory demonstrated the tendency of the isolated C2 domain of PKCε to preferentially bind phospholipid vesicles containing phosphatidic acid (Garcia-Garcia *et al*., 2001; Ochoa *et al*., 2001). The results obtained above also suggest that the full-length enzyme needs a continuous source of PtdOH to translocate to the plasma membrane in physiological conditions. Because exogenous PtdOH (DiC8-PtdOH) can be added to the cell culture medium and is incorporated rapidly into cell membranes where it subsequently participates in cellular functions (Fang *et al*., 2001), we tested the ability of this soluble-permeant phosphatidic acid to activate both wild-type and the different mutants. When cells transfected with PKCε-GFP were stimulated with 22 μg/ml PtdOH (Figure 7A), maximal plasma membrane localization was reached 60 s after PtdOH addition and the protein remained located in the plasma membrane for the 600 s the experiment lasted ($R = 0.82 \pm 0.07$). However, when cells transfected with PKCε-I89N-GFP were stimulated with PtdOH, a maximal translocation of $R = 0.55 \pm 0.03$ was observed 89 s after stimulation. A more drastic effect was obtained when the cells were transfected with PKCε-W23A/R26A/R32A-GFP mutant, when $R = 0.44 \pm 0.01$ was the maximal translocation ratio obtained 136 s after stimulation with PtdOH. To test whether these results were concentration dependent, we also studied the effect of a lower concentration of PtdOH (11 μg/ml) on the plasma membrane localization of both wild-type and mutants (Figure 7B). In this case, the rate of localization of PKCε-GFP slowed down, maximal localization ($R = 0.84 \pm 0.08$) being obtained 119 s after PtdOH stimulation. A more drastic effect was obtained when the cells were transfected with PKCε-W23A/R26A/R32A-GFP and PKCε-I89N-GFP mutants, when maximal translocations to the plasma membrane were $R = 0.42 \pm 0.05$ and 0.54 ± 0.11 224 and 179 s after stimulation, respectively.

These data confirm that PtdOH is able to induce the translocation of the enzyme to the plasma membrane. Furthermore, the translocation profiles of both wild-type and mutant proteins were more similar to those exhibited by cells activated by antigen than the profiles occurring when the cells were stimulated by DiC8. The more drastic effect of the C2 domain mutations on protein localization observed after IgE receptor stimulation could be due to the lower levels of phosphatidic acid generated into the cells physiologically. In fact, it has been described that the normal molar concentration of PtdOH in cell membranes is <5% of that of PC (Buckland and Wilton, 2000).

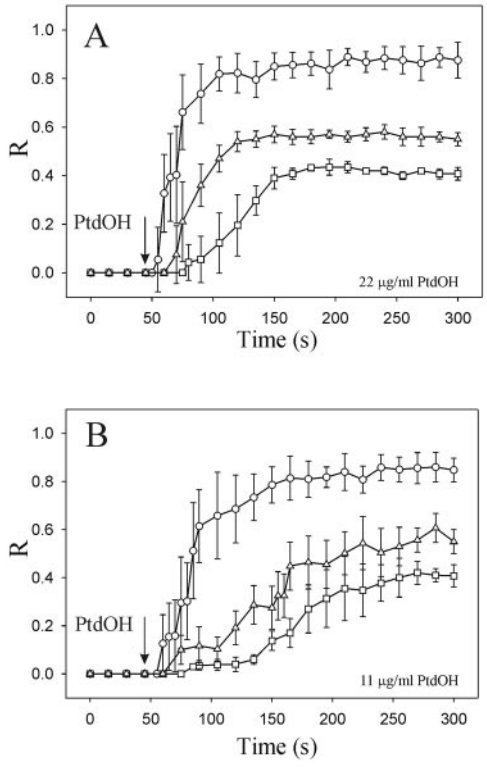

Figure 7. PtdOH-dependent plasma membrane localization of wild-type PKCε (○), PKCε-W23A/R26A/R32A (□) and PKCε-I89N (△). Averages of the time courses of localization when the cells transfected with the different constructs were stimulated with 22 μg/ml PtdOH (A) and 11 μg/ml PtdOH (B).

PKCε Is Activated by PA In Vitro

We also studied the ability of PKCε to phosphorylate a specific substrate in vitro under PtdOH-dependent conditions. For this, HEK293 cells were transfected with the same constructs as mentioned above, the only difference being that they were cloned in a vector, which enabled us to express them, fused to a hemagglutinin tag. After purification of the wild-type PKCε and the different mutants, we studied the PtdOH dependence of enzyme activation in the absence and in the presence of DAG (Figure 8A) by using histone III-S as a substrate. It is interesting to note that under the conditions used in these experiments, no basal specific activity was detected when the phospholipid vesicles contained 5 or 10 mol% DOG in the absence of POPA. Interestingly, Figure 8A also shows that it was necessary to include DAG in the lipid vesicles to observe a POPA-dependent activation, suggesting that at least in vitro both PtdOH and DAG interactions with PKCε are necessary for the full activation of the enzyme.

In addition, we studied the effect of the different substitutions of the amino acidic residues in loops 1 and 3 on the POPA-dependent activation of the enzyme (Figure 8B). As shown, the more drastic effect was once again observed by the mutations performed in loop 1, where 80 mol% of POPA in the lipid vesicles only produced 50% of the catalytic activity of the enzyme. However, the mutants containing the substitutions affecting loop 3 were activated almost identically to the wild-type protein.

Because the plasma membrane also contains phosphatidylserine constitutively, we included in the lipid mixture 20 mol%

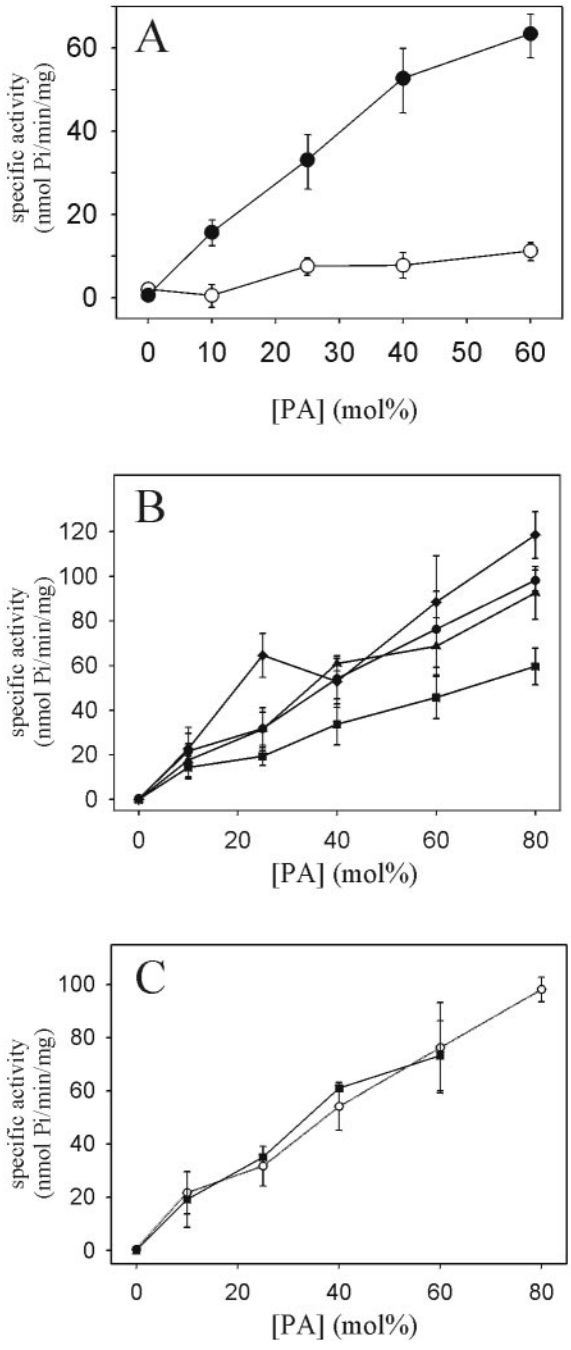

Figure 8. (A) Specific activity of PKCε by using large unilamellar vesicles containing POPC/POPA (99-X: X, with X being the molar fraction of POPA in each case) (○) and POPC/DOG/POPA (95-X:5:X, with X being the molar fraction of POPA in each case) (●). (B) PtdOH-dependent activation of wild-type PKCε (●), PKCε-W23A/R26A/R32A (■), PKCε-I89N (▲), and PKCε-Y91A (♦) in large unilamellar vesicles. PKC activity was measured using POPC/DOG/POPA large unilamellar vesicles (95-X:5:X, with X being the molar fraction of POPA in each case). (C) Specific activity of PKCε by using large unilamellar vesicles containing POPC/POPA (99-X: X, with X being the molar fraction of POPA in each case) (○) (these results are the same that in part B of this figure, and have been represented here to facilitate the comparison of the data) and POPC/POPS/DOG/POPA (95-X:20:5:X, with X being the molar fraction of POPA in each case) (■). The total lipid concentration was 0.2 mM and Histone II-SS was used as a substrate. Error bars indicate the SEM for triplicate determinations.

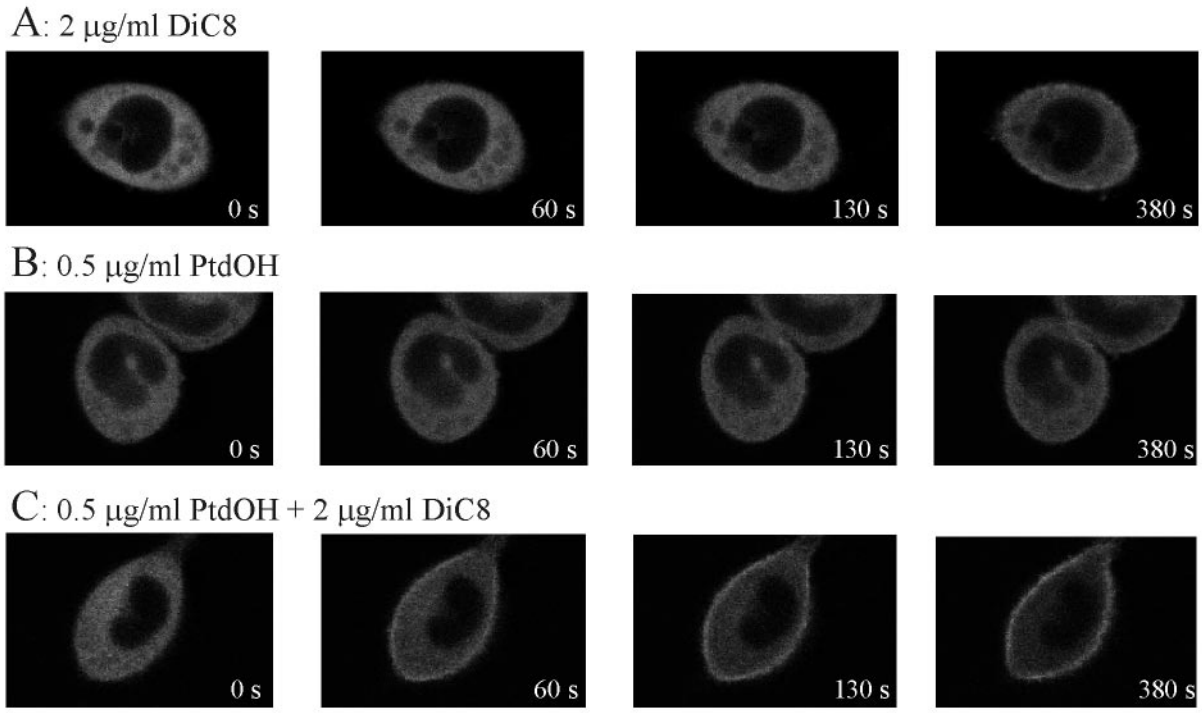

Figure 9. RBL-2H3 cells were stimulated with 2 μg/ml DiC8 (A), 0.5 μg/ml PtdOH (B), or a combination of the two activators together, 2 μg/ml DiC8 + 0.5 μg/ml PtdOH (C). Time series were collected each 5 s during 10 min. These cells are representative of three independent experiments.

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), which is assumed to be a physiological concentration in the plasma membrane (McMurray, 1973). Thus, the vesicles used to measure the specific activity of the wild-type PKCε contained POPC/POPS/DOG and increasing concentrations of POPA. As shown in Figure 8C, the POPA-dependent activity of the enzyme was similar both in the presence and in the absence of POPS in the phospholipid vesicles and no additive effect was observed, suggesting that POPA is a more specific activator than phosphatidylserine under these conditions.

PtdOH and DAG Exhibit Synergistic Effects on the Translocation of PKCε to the Plasma Membrane

Together, the results obtained in this work strongly suggest that both PtdOH and DAG generated in the plasma membrane upon activation of the IgE receptor in RBL-2H3 cells, are responsible for the localization of PKCε in the membrane and for its activation. To further test this hypothesis, we promoted the plasma membrane localization of PKCε by adding exogenous applications of low concentrations of PtdOH and DAG. Thus, when 2 μg/ml DiC8 was used to stimulate RBL-2H3 cells transfected with PKCε-GFP, no translocation of the enzyme was detected, only and small and localized amount of protein was observed in the plasma membrane 380 s after stimulation (Figure 9A). Similar results were obtained when the cells transfected with PKCε-GFP were stimulated with 0.5 μg/ml PtdOH (Figure 9B). However, when the cells were stimulated with the two agents together (2 μg/ml DiC8 + 0.5 μg/ml PtdOH), a partial PKCε-GFP localization was observed 60 s after stimulation ($R = 0.48$) that was increasing until $R = 0.6$ at 130 s and $R = 0.78$ at 380 s (Figure 9C). These results further support the hypothesis proposed previously where simultaneous production of PtdOH and DAG is essential for the PKCε localization in the plasma membrane upon activation of the IgE receptor.

DISCUSSION

PKC activation is known to play a role in the differentiation and proliferation of various cell types (Newton, 2001). PKC isoenzymes differ with regard to tissue distribution, regulation, and enzymatic properties, and the vigorous efforts of various groups are beginning to clarify the different biological roles of individual isoenzymes. Recent studies have suggested that the C2 domain of PKCε might interact with acidic phospholipids through several amino acidic residues located at the top of the molecule (Ochoa *et al*., 2001). However, there is no biological evidence to support a specific function for this C2 domain that depends on lipids as direct activators or regulators of the enzyme. In this work, we have studied the mechanism by means of which full-length PKCε interacts with the plasma membrane under physiological conditions, demonstrating that the C2 domain of PKCε is an important and critical player in the biological membrane localization and activation of the enzyme.

Physiological and Molecular Mechanism for PKCε Membrane Targeting

Some of the most interesting aspects of PKCε localization to emerge from this study are the profiles obtained when the cells were transfected with the wild-type and the different mutant proteins and stimulated with antigen. These experiments demonstrate that the residues located in loops 1 and 3 of the C2 domain directly or indirectly participate in the localization of the enzyme in the plasma membrane. Furthermore, these results suggest that the diacylglycerol generated by receptor activation is not the only force anchoring the enzyme in the plasma membrane because the translocation profiles obtained when cells (transfected with wild-type and mutant enzymes) were stimulated only with permeable DiC8 did not correlate

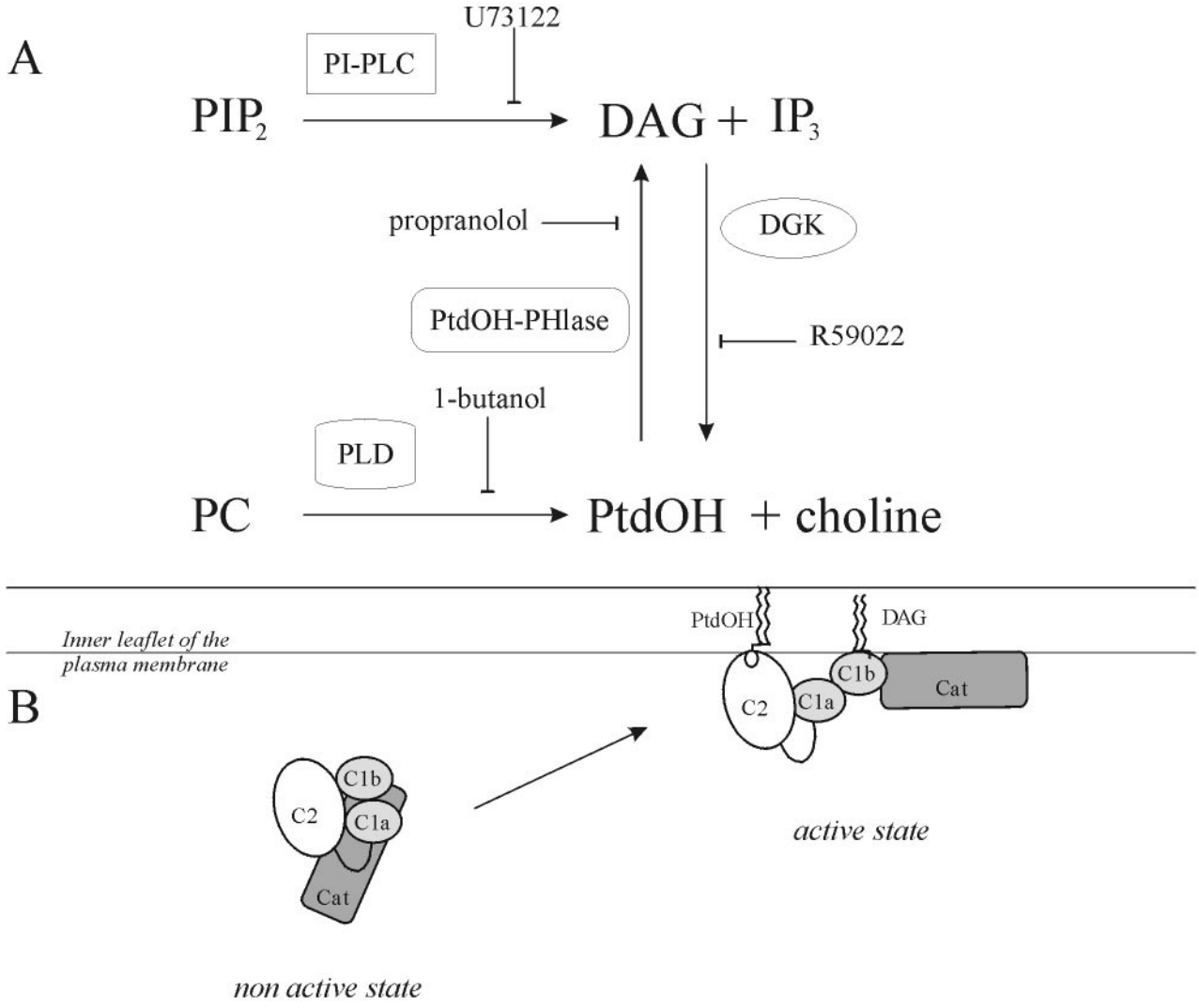

Figure 10. Mechanism of activation of PKCε in RBL-2H3 cells upon activation of the IgE receptor. (A) Scheme showing the lipid intermediate generated upon receptor stimulation. In a first step, phosphatidylinositol-4,5-bisphosphate is hydrolyzed to produce DAG and inositol triphosphate by PI-PLC; simultaneously PC is hydrolyzed by PLD to produce PtdOH and choline. In a second step, DAG and PtdOH generated are transformed to PtdOH and DAG, respectively, by the action of DGK and PtdOH-phosphohydrolase. The inhibitors of each reaction are marked with a bar arrow. (B) Together, the results obtained in this work suggest that when the IgE receptor is activated, the enzyme translocates to the plasma membrane when PtdOH and DAG are generated simultaneously in the plasma membrane, thus leading to the catalytic activation of the enzyme (active state). It has been shown the C1b domain as DAG receptor assuming the model proposed by Szallasi *et al.* (1996).

well with the profiles obtained after physiological stimulation of the FcεRI (IgE receptor) (compare Figures 3B and 4B).

To get further insights into the pathways involved in the PKCε membrane localization, which is dependent on IgE receptor stimulation, we used a collection of specific inhibitors that block the different phospholipases activated and that, in turn, generate both PtdOH and DAG. The fact that the inhibition of PI-PLC or PLD led to the total inhibition of PKCε membrane localization and that the inhibition of DGK led only to a partial localization of the enzyme leads us to postulate the following sequence of events (Figure 10A): antigen cross-linking of the IgE receptor activates both PI-PLC and PLD and generates both DAG and PtdOH, respectively, which are essential for enzyme localization; these two second messengers produced might be further metabolized in a second step in which DAG is transformed into PtdOH by DGK and, the PtdOH generated by PLD is transformed into DAG by phosphatidate phosphohydrolase, thus maintaining the equilibrium of activators between DAG and PtdOH. This way of extent the existence of both activators in the plasma membrane acting on the same PKCε molecule would increase the time that the enzyme is localized in the plasma membrane and prolong enzyme activation, explaining the slow membrane dissociation exhibited by PKCε when the cells were activated with antigen (Figure 2B).

The partial inhibition of PKCε localization obtained after using DGK inhibitor II further supports the hypothesis that PtdOH generated by PLD together with the DAG generated by PI-PLC in a first step is essential for the membrane targeting. This is lent weight by the fact that both enzymes were activated

and partial membrane targeting was observed. The partial inhibition also confirms the idea that DGK generates PtdOH, which is needed to fully localize the enzyme in the plasma membrane, although to a lesser extent than the PtdOH generated by PLD (Figure 10A). Few conclusions can be obtained from the use of propranolol except that the results reinforce the hypothesis that the lack of both PtdOH and DAG impedes the proper localization of the enzyme. This is due to the double action mechanism of propranolol because both the PtdOH and the phosphohydrolase are blocked by the drug (Koul and Hauser, 1987).

Together, the cell localization and in vitro kinase assays performed in this study suggest a model for the physiological activation of PKCε, whereby the enzyme can anchor in the membrane either by means of the C2 domain or alternatively through the C1 domain if PtdOH or DiC8, respectively, are present at high concentrations. When PtdOH and DiC8 are present in the plasma membrane at very low concentrations, which seems to be the case under physiological conditions, both messengers need to act together and they can potentiate the effect of each other, leading to PKCε localization in the plasma membrane. Additionally, full activation of the catalytic activity of the enzyme is only reached when both activators are present in the plasma membrane and bound to the enzyme, thus conferring its active state conformation (Figure 10B).

It has been shown that PKCα can colocalize with PLD and DGK, suggesting an activating role in the case of PLD and an inhibitory effect in the case of DGK (Slaaby *et al*., 2000; Powner *et al*., 2002; Luo *et al*., 2003). Nevertheless, there is no evidence of the colocalization of PLD, DGK, and PKCε to date. Taking into account that the spatio-temporal membrane localization of PKCε (1.5 min) is a slower process than for PKCα (2–26 s) (Bolsover *et al*., 2003), these results would fit a sequential model whereby, after IgE receptor stimulation of RBL-2H3 cells, PKCα would translocate first to the plasma membrane, where it would activate PLD, which in turn would promote PKCε membrane localization and activation in a PtdOH-dependent manner. Unfortunately, the function of PKCε upon activation of the FcεRI is still far from clear. Early evidence suggested that this enzyme activates the AP-1 transcription factor, leading to an increase in cell proliferation (Razin *et al*., 1994; Chang *et al*., 1997), but no direct correlation with degranulation has been found when RBL-2H3 cells are transfected with this isoenzyme (Chang *et al*., 1997; our unpublished observations). The new findings presented in this work place PKCε in a new scenario, together with PLD and probably DGK as well. A recent publication has shown very nicely that a continuous source of PtdOH generated by PLD is essential for antigen-stimulated membrane ruffling in mast cells because it regulates phosphatidylinositol 4,5-bisphosphate synthesis (O'Luanaigh *et al*., 2002), more specifically, by activating type I phosphatidylinositol 4-phosphate 5-kinase (PIP5) kinase, which can be stimulated indirectly by PtdOH derived from PLD activation (Honda *et al*., 1999) or directly by ARF proteins (Jones *et al*., 2000a). Whether PKCε is directly involved in this activation process is still unknown; however it might serve as a link between the PtdOH generated and some additional intermediate, which would lead to the activation of type I PIP5 kinase.

Recent reports have demonstrated two important functions for the PtdOH generated by PLD isoenzymes. One of these functions is the recruitment of cytosolic proteins to the membrane and the second is as a regulator in the activation of different proteins and lipid kinases (see Cockcroft, 2001; Andresen *et al*., 2002 for extensive reviews). The direct interaction with cellular proteins and/or stimulation by PtdOH have been demonstrated in the cases of the protein kinase cRaf-1 (Ghosh *et al*., 1996), type I PIP5 kinase (Jenkins *et al*., 1994; Jones *et al*., 2000b), the mammalian target of rapamycin (mTOR) (Fang *et al*., 2001), cAMP-specific phosphodiesterase PDE4D3 (Grange *et al*., 2000), tyrosine protein phosphatase SHP-1 (Frank *et al*., 1999) and the $p47^{phox}$ subunit of phagocyte NADPH oxidase (Karathanassis *et al*., 2002). The results obtained in this work suggest that PKCε can be included in this family as well.

In conclusion, this work defines a new role for the PtdOH generated by IgE receptor stimulation: the membrane localization and activation of PKCε. A new model for the enzyme's activation has been proposed, where, importantly, the C2 domain plays a key role in both localization and activation. Further studies to identify downstream targets of this specific PKC isoenzyme are under way and will hopefully be of great help in unveiling this complex puzzle.

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