

Phosphatidylinositol 4,5-Bisphosphate Anchors Cytosolic Group IVA Phospholipase A₂ to Perinuclear Membranes and Decreases Its Calcium Requirement for Translocation in Live Cells

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The eicosanoids are centrally involved in the onset and resolution of inflammatory processes. A key enzyme in eicosanoid biosynthesis during inflammation is group IVA phospholipase A₂ (also known as cytosolic phospholipase A_{2α}, cPLA_{2α}). This enzyme is responsible for generating free arachidonic acid from membrane phospholipids. cPLA_{2α} translocates to perinuclear membranes shortly after cell activation, in a process that is governed by the increased availability of intracellular Ca²⁺. However, cPLA_{2α} also catalyzes membrane phospholipid hydrolysis in response to agonists that do not mobilize intracellular Ca²⁺. How cPLA_{2α} interacts with membranes under these conditions is a major, still unresolved issue. Here, we report that phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] promotes translocation of cPLA_{2α} to perinuclear membranes of intact cells in a manner that is independent of rises in the intracellular Ca²⁺ concentration. PtdIns(4,5)P₂ anchors the enzyme to perinuclear membranes and allows for a proper interaction with its phospholipid substrate to release arachidonic acid.

INTRODUCTION

It is well established that the hydrolytic attack of arachidonic acid (AA)-containing phospholipids by cytosolic phospholipase A_{2α} (cPLA_{2α}) constitutes the first regulatory step of the eicosanoid biosynthetic cascade (Bonventre, 2004; Hirabayashi *et al.*, 2004). cPLA_{2α} is unique among all the phospholipase A₂ family members (Six and Dennis, 2000) due to its substrate selectivity for AA-containing phospholipids and its tight regulation by Ca²⁺ and phosphorylation (Clark *et al.*, 1991; Lin *et al.*, 1993; Dessen *et al.*, 1999; Gijón *et al.*, 2000a). Free AA generated by activated cPLA_{2α} can be oxygenated into a variety of compounds, called the eicosanoids, that not only are involved in acute and chronic inflammatory diseases such as arthritis, asthma, and post-ischemic tissue-injury but also regulate physiological processes such as renal function and female reproductive events, including parturition (Bonventre, 1997, 2004; Uozumi *et al.*, 1997; Hirabayashi *et al.*, 2004).

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Abbreviations used: AA, arachidonic acid; [Ca²⁺]_i, intracellular Ca²⁺ concentration; cPLA_{2α}, cytosolic group IVA phospholipase A₂; PtdIns, phosphatidylinositol.

cPLA_{2α} is a cytosolic enzyme in resting cells and translocates to membranes when cell activation takes place. The enzyme possesses a calcium binding domain (C2) in its N-terminal half that helps the enzyme interact with membrane phospholipid when the intracellular Ca²⁺ concentration ([Ca²⁺]_i) rises. A single mutation in this domain, i.e., substitution of Asp-43 with Asn, completely abrogates the Ca²⁺-dependent translocation of cPLA_{2α} to cellular membranes and, as a result, AA release is inhibited (Qiu *et al.*, 1998; Gijón *et al.*, 1999; Perisic *et al.*, 1999).

However, stimuli that are known not to affect [Ca²⁺]_i, such as bacterial lipopolysaccharide, phorbol esters or okadaic acid, are nonetheless able to induce robust AA mobilization and eicosanoid production responses in a cPLA_{2α}-dependent manner (Shinohara *et al.*, 1999; Gijón *et al.*, 2000b). The mechanism by which cPLA_{2α} interacts with membranes under conditions that do not involve increase of [Ca²⁺]_i has remained unaddressed.

In vitro activity studies carried by Leslie and Channon (1990) in the early 1990s using a partially purified cPLA_{2α} preparation had suggested that anionic phospholipids such as phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] and phosphatidic acid, when incorporated into the vesicle substrate, were capable of stimulating the activity of the enzyme and decreasing its Ca²⁺ requirement from millimolar to nanomolar levels. More recently, Mosior *et al.* (1998) described the potent and specific increase in affinity of pure cPLA_{2α} for surfaces containing PtdIns(4,5)P₂ at physiological concentrations, and this effect paralleled an increase in substrate hydrolysis. Importantly, the enhancing effects of PtdIns(4,5)P₂ were observed even in the presence of EGTA ([free Ca²⁺] < 2 nM). Das and Cho (2002) recently described a cluster of cationic residues in the catalytic domain of

cPLA₂ α (Lys⁴⁸⁸, Lys⁵⁴¹, Lys⁵⁴³, and Lys⁵⁴⁴ in the human sequence) that seem to play a role in the *in vitro* activation of the enzyme by PtdIns(4,5)P₂, a finding that was corroborated later on by Six and Dennis (2003).

With regard to intact cells, we observed that in UV light-treated macrophages, increased PtdIns(4,5)P₂ synthesis occurs at resting Ca²⁺ levels, and this parallels the cPLA₂ α -dependent mobilization of AA (Balsinde *et al.*, 2000). These data led us to propose that PtdIns(4,5)P₂ might be involved in cPLA₂ α activation at Ca²⁺ levels equaling those pertaining to unstimulated cells (Balsinde *et al.*, 2000). In this article, we demonstrate that PtdIns(4,5)P₂ anchors cPLA₂ α to perinuclear membranes of intact cells in a manner that is independent of rises in [Ca²⁺]_i, thus allowing for proper interaction of the enzyme with its phospholipid substrate to optimally release AA.

MATERIALS AND METHODS

Reagents

Cell culture media and supplements were purchased from Invitrogen (Paisley, Scotland). BODIPY^{TR}-X C₆-PI(4,5)P₂, BODIPY^{TR}-X C₆-PI(3,4)P₂, carriers (neomycin and histone), and Fluo-3-AM were purchased from Invitrogen (Carlsbad, CA). Other reagents were from Sigma Ibérica (Madrid, Spain).

Lipid Preparation

Lipids were prepared following the manufacturer's instructions. Briefly, 2 μ g of phospholipid was mixed with 2 μ l of carrier (neomycin or histone, 0.5 mM); resuspended in 200 μ l of Hank's balanced salt solution (HBSS) containing 10 mM HEPES, and unless otherwise indicated, 1.3 mM CaCl₂; sonicated in a water bath for 2 min; and allowed to rest at 37°C for 10 min before use. Final concentration of phosphatidylinositol bisphosphate (PtdInsP₂) in the solution is 5.7 μ M.

Cells

Human embryonic kidney (HEK) cells were cultured in DMEM supplemented with 2 mM glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. Cells were passaged twice a week by trypsinization. Cells (40–70% confluence) were transfected with 1 μ g of plasmid/ml using LipofectAMINE Plus (Invitrogen), following the manufacturer's instructions. For stably transfected cells, 1 mg/ml G418 was used for selection and subsequent passages. RAW 264.7 cells were cultured in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO₂ humidified incubator.

Constructs

The DNA sequence of human cPLA₂ α was cloned into the pEGFP vector (BD Biosciences Clontech, Palo Alto, CA) using Hind-III and Pst-I cloning sites. This construct codes for the expression of a fusion protein containing an N-terminal enhanced green fluorescent protein (EGFP) followed by the entire sequence of the human cPLA₂ α . Wild-type cPLA₂ α was mutagenized within the C2 domain by replacing Asp⁴³ with Asn (D43N), using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotides 5'-CATGCTTGATACCCAAATCCCTATGTGGAAC-3' (forward) and 5'-GTCCACATAGGGATTGGAGTATCAAGCATG-3' (reverse). Mutagenesis was confirmed by sequencing. Wild type EGFP-cPLA₂ α was also mutagenized to produce EGFP-K488E/K541A/K543A/K544A-cPLA₂ α in two steps. First, the mutant EGFP-K541A/K543A/K544A-cPLA₂ α was obtained by using the QuikChange XL site-directed mutagenesis kit (Stratagene) and the oligonucleotides 5'-GAGCCTTGGATGTCGCAAGTGCAGCGAT-TCACTAGTGGACAG-3' (forward) and 5'-CTGTCCACTACATGAATCG-CTGCACTTGGACATCCAGAGGCTC-3' (reverse). Then, a second round of mutagenesis was performed to change Lys⁴⁸⁸ to Glu using the primers 5'-GGACGTGCTGGGGAGGTACACAACCTCATGC-3' (forward) and 5'-GCATGAAGTTGTACTCCCAAGCAGCTCC-3' (reverse).

Confocal Microscopy

Cells were seeded on glass-bottom culture dishes (MatTek, Ashland, MA) coated with poly-L-lysine (Sigma Ibérica). After 24 h, the culture medium was replaced with HBSS containing 10 mM HEPES and 1.3 mM CaCl₂. Cells were monitored at 37°C by confocal microscopy using a laser scanning Radiance 2100 system (Bio-Rad, Hercules, CA) coupled to a Nikon TE-2000U inverted microscope equipped with a DH-35 tissue culture dish heater (Warner Instruments, Hamden, CT). Images were obtained with a CFI Plan Apo 60 \times , oil immersion, 1.40 numerical aperture objective, which provided a theoretical

confocal layer thickness of \sim 0.4 μ m at the wavelengths used. Green fluorescence from the EGFP was monitored at 488-nm argon laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass blocking filters. Red fluorescence from BODIPY-TRx was monitored at 543-nm HeNe laser excitation using a HQ590/570 long band pass blocking filter. In some experiments, cells were incubated with HBSS without calcium and then discrete amounts of CaCl₂ were added sequentially to obtain extracellular concentrations of 6, 15, 30, 60, 150, and 300 μ M.

AA Release

HEK cells (2.5 \times 10⁵/well) were labeled with 0.5 μ Ci of [³H]AA for 20 h. Cells were then extensively washed and overlaid with 0.5 ml of serum-free DMEM supplemented with 0.1 mg/ml albumin and treated with 5 μ M thimerosal for 15 min to blunt fatty acid reacylation (Pérez *et al.*, 2004). Cells were then treated with PtdInsP₂, and the supernatants were collected at different time points. After extraction, lipids were separated by thin layer chromatography using the system n-hexane/diethyl ether/acetic acid (70:30:1 by volume). Spots corresponding to AA and phospholipid were scraped, and radioactivity was quantified by liquid scintillation counting.

Intracellular Ca²⁺ Measurements

HEK cells were loaded with 3 μ M Fluo-3-AM for 20 min in medium with 10% serum at 37°C in a 5% CO₂ incubator. Cells were then washed and incubated with HBSS containing 10 mM HEPES and 1.3 mM CaCl₂. Fluorescence was monitored under confocal microscope at 488 laser excitation and the combination of a HQ500 long band pass and a HQ560 short band pass blocking filters, having the iris totally open. At the end of each experiment calibration was done as previously described by Kao *et al.* (1989), using MnCl₂ at a final concentration of 2 mM, and lysis with 0.05% saponin to obtain the background signal. Where analysis of intracellular calcium levels was studied in the presence of different concentration of extracellular CaCl₂, cells were loaded with Fluo-3, washed with HBSS with 2 mM EGTA, and layered with HBSS containing 10 mM HEPES in the absence of CaCl₂. Different amounts of CaCl₂ were added every 10 min, and fluorescence was recorded every 15 s under a confocal microscopy. Calibration was also performed at the end of each experiment as indicated above.

RESULTS AND DISCUSSION

We were interested in examining whether cellular increases in PtdIns(4,5)P₂ regulate cPLA₂ α translocation to intracellular membranes in live cells. To this end, we prepared HEK cells stably expressing a fusion protein of EGFP and human cPLA₂ α . The cells were studied under the confocal microscope during treatment with fluorescent PtdIns(4,5)P₂ [BODIPY^{TR}-tagged PtdIns(4,5)P₂; abbreviated as TR-PI(4,5)P₂]. When complexed with histone or neomycin carriers that counteract the negative charges of TR-PI(4,5)P₂ and make it membrane permeable (Ozaki *et al.*, 2000), it takes 5–10 min for the phospholipid to penetrate into the cell (Figure 1). Once inside, TR-PI(4,5)P₂ localized primarily to perinuclear membranes, in accord with previous observations (Ozaki *et al.*, 2000). Movement of TR-PI(4,5)P₂ to perinuclear membranes was closely followed by the complete translocation of cPLA₂ α to the same location (Figure 1A). When EGFP-cPLA₂ α transfected cells were treated only with the carrier, EGFP-cPLA₂ α did not translocate (Figure 1B). Furthermore, when cells transfected with EGFP alone were exposed to TR-PI(4,5)P₂, the green fluorescence pattern did not change (Figure 1C). AA mobilization was studied under these conditions. Figure 1D shows that exposure of the cells to TR-PI(4,5)P₂ enhanced AA release, thus indicating that membrane phospholipid hydrolysis follows from translocation of cPLA₂ α to perinuclear membranes.

A possible explanation for TR-PI(4,5)P₂-induced cPLA₂ α membrane translocation and subsequent activation could be that the phosphoinositide, once inside the cell, is hydrolyzed by phospholipase C to generate inositol 1,4,5-trisphosphate, which results in elevated [Ca²⁺]_i and hence cPLA₂ α translocation. However, cPLA₂ α translocation to membranes was also observed when the cells were treated with TR-PI(3,4)P₂, whose hypothetical hydrolysis by phospholipase C would not yield inositol 1,4,5-trisphosphate (Figure 1E). That both

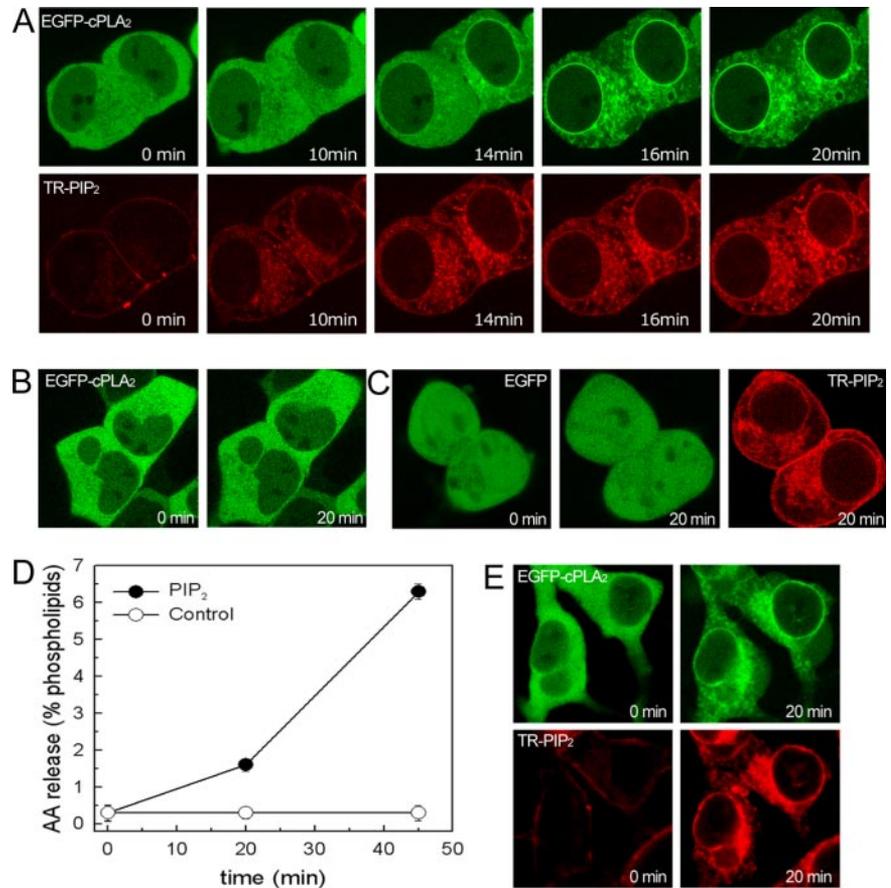


Figure 1. EGFP-cPLA₂α translocation and AA release driven by TR-PIP₂. EGFP-cPLA₂α (A, B, and E) or EGFP (C)-transfected HEK cells were treated with TR-PI(4,5)P₂/histone (A and C), TR-PI(3,4)P₂/histone (E), or carrier only (histone; B). Pictures were taken at the indicated time points under the confocal microscope. (D) [³H]AA-labeled HEK cells were either untreated (open circles) or treated with TR-PI(4,5)P₂/carrier (closed circles), and [³H]AA release was measured at the indicated time periods. Each experiment was performed at least three times with identical results.

PtdIns(4,5)P₂ and PtdIns(3,4)P₂ isomers modify the subcellular localization of cPLA₂α suggests a charge effect rather than the result of a metabolic transformation via phospholipase C. The PtdIns(3,4)P₂-induced translocation of cPLA₂α was accompanied by an increased release of AA, similar in extent to that produced by PtdIns(4,5)P₂ (our unpublished data). This is consistent with the *in vitro* studies of Six and Dennis (2003) showing increased cPLA₂α activity in response to PtdIns(3,4)P₂. Interestingly, Das and Cho (2002) failed to observe an effect of PtdIns(3,4)P₂ on the activity of cPLA₂α. The reasons for this discrepancy may likely arise from differences in the experimental protocols followed by these authors.

Experiments were also conducted with the macrophage cell line RAW 264.7. Owing to their immunoinflammatory nature, RAW 264.7 macrophages possess a robust machinery for eicosanoid biosynthesis and thus provide a more appropriate cell context to validate cPLA₂α translocation and AA release in response to PtdIns(4,5)P₂. Exposure of EGFP-cPLA₂α-transfected macrophages to TR-PI(4,5)P₂ resulted in membrane translocation of the enzyme (Figure 2A) and enhanced AA release (Figure 2B).

We also performed measurements with pure PtdIns(4,5)P₂, isolated from bovine brain. Probably because of the presence of long-chain fatty acids in pure PtdIns(4,5)P₂, it was not possible for us to deliver this phospholipid into the cell's interior by using a shuttle. Thus, for these experiments we used digitonin-permeabilized cells (Balsinde *et al.*, 2000). Using this procedure, we confirmed that native PtdIns(4,5)P₂ induced translocation of EGFP-cPLA₂α to perinuclear membranes in a similar manner to that previously observed with BODIPY-TR-PI(4,5)P₂ in Figure 1 (our unpublished data).

Mosior *et al.* (1998) have shown that binding of cPLA₂α to phospholipid vesicles enriched in PtdInsP₂ is essentially a Ca²⁺-independent process. To verify whether this is also the case in live cells, we incubated the cells in a Ca²⁺-free EGTA-containing buffer. The continued presence of cells in Ca²⁺-free buffer lowers the intracellular Ca²⁺ level well below that normally occurring in resting cells incubated in a regular Ca²⁺-containing buffer (Di Virgilio *et al.*, 1984). Under these Ca²⁺-free conditions, no translocation of cPLA₂α was observed after addition of TR-PI(4,5)P₂, even though the phosphoinositide penetrated normally into the cell (Figure 3). Interestingly, if Ca²⁺ was restored to the incubation medium, a very rapid distribution of cPLA₂α to the perinuclear membranes of TR-PI(4,5)P₂-treated cells was observed (Figure 3). These results suggest that, in cells, the PtdInsP₂ effect is not Ca²⁺-independent; rather, a threshold Ca²⁺ is necessary for cPLA₂α to translocate to membranes. This view is consistent with previous data by Das and Cho (2002).

To further characterize this Ca²⁺ effect, we conducted experiments with a mutant construct where the cPLA₂α has the mutation D43N (EGFP-D43N-cPLA₂) which severely limits the ability of the enzyme to bind calcium (Bittova *et al.*, 1999; Gijón *et al.*, 1999; Perisic *et al.*, 1999). As shown in Figure 4, the mutant EGFP-D43N-cPLA₂ did not translocate in response to TR-PI(4,5)P₂, even in the presence of 1.3 mM CaCl₂ in the incubation buffer, demonstrating that a functional C2 domain is necessary for cPLA₂α translocation to perinuclear structures in response to PtdInsP₂.

Because TR-PI(4,5)P₂ and cPLA₂α seem to move to similar intracellular structures (Figures 1–3), a colocalization analysis was performed. Figure 5A shows the merge of green

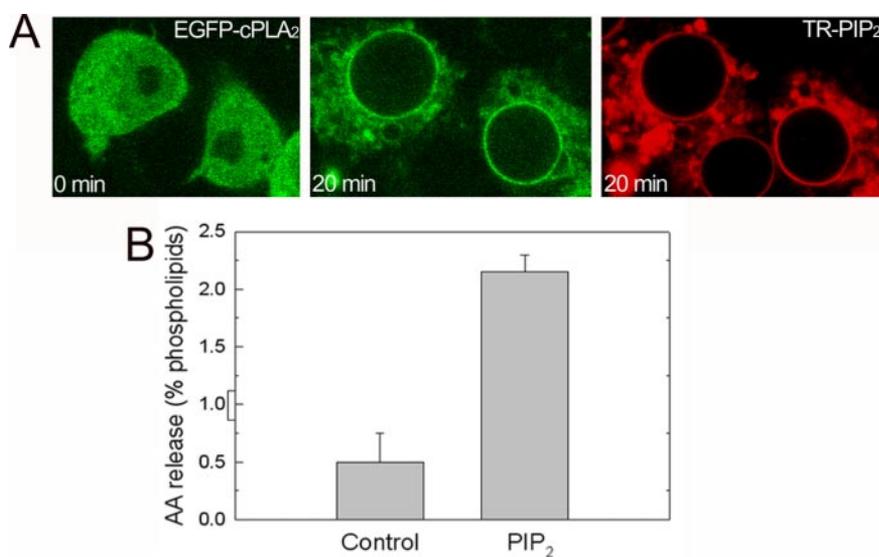


Figure 2. TR-PIP₂ effects on RAW 264.7 cells. RAW 264.7 macrophages transfected with EGFP-cPLA₂ were treated with TR-PI(4,5)P₂/histone for the indicated periods of time and analyzed by confocal microscopy (A) or for [³H]AA release (B) as in Figure 1. Each experiment was performed at least three times with identical results.

(from the EGFP-cPLA₂α) and red [from the TR-PI(4,5)P₂] fluorescences in cells treated for 20 min with TR-PI(4,5)P₂, suggesting colocalization in the perinuclear region. Analysis of colocalization with the LaserPix confocal image software (Bio-Rad) provides a better definition of the pixels showing perfect colocalization of both fluorescences (Figure 5B, white mask). In Figure 5C, a three-dimensional representation of such colocalization is shown, and the color of each pixel defines the level of colocalization. Nuclear membrane and perinuclear structures (most likely Golgi and endoplasmic reticulum) show the most intense colocalization. As a control for the colocalization analyses, note a cell in the bottom left corner of Figure 5, A–C, that does not express EGFP-cPLA₂α and thus stains only red, and another cell in the bottom right corner that expresses the EGFP-cPLA₂α but did not take TR-PI(4,5)P₂ in, and thus stains only green. Accordingly, these two cells do not show in the colocalization mask (Figure 5B) or in the three-dimensional representation (Figure 5C).

Taking advantage of the fact that in the absence of extracellular calcium, TR-PI(4,5)P₂ fails to induce EGFP-cPLA₂α

translocation to membranes (see above), green and red fluorescences can be analyzed in the same cell at different localizations (Figure 5D). Under these conditions, the colocalization coefficients obtained with the LaserPix software were red to green, 0.37 ± 0.08; and green to red, 0.33 ± 0.06. After restoring calcium to the extracellular medium, the colocalization analysis was repeated on the same cells and the data were red to green, 0.40 ± 0.05; and green to red, 0.88 ± 0.01.

Together, the results presented so far indicate that PtdIns(4,5)P₂ functions to anchor cPLA₂α to the perinuclear and nuclear membranes in live cells, by a process that requires the presence of a certain threshold level of Ca²⁺ as well as a functional Ca²⁺ binding site in the enzyme (Figure 3). Thus, PtdIns(4,5)P₂ may either provide a second binding site for cPLA₂α in addition to the Ca²⁺ binding site or act to lower the Ca²⁺ requirement of cPLA₂α for membrane binding. To evaluate these two possibilities, changes in [Ca²⁺]_i were monitored in Fluo-3-loaded cells exposed to the phosphoinositide (Figure 6). TR-PI(4,5)P₂ induced changes in [Ca²⁺]_i with a very different pattern, potency, and kinetics from the calcium ionophore ionomycin, which is also known

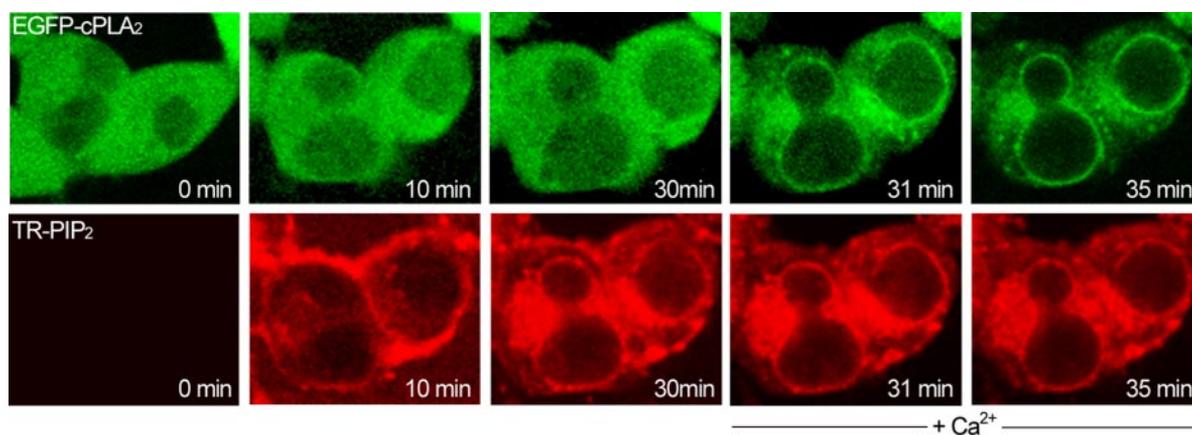
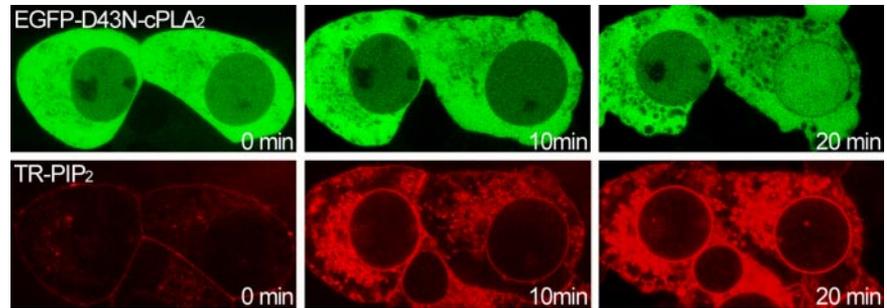


Figure 3. Ca²⁺ is necessary for EGFP-cPLA₂α translocation in response to TR-PI(4,5)P₂. EGFP-cPLA₂-transfected HEK cells were treated with TR-PI(4,5)P₂/histone in HBSS medium without Ca²⁺, and pictures were taken under the confocal microscope. After a 30-min treatment, 1.3 mM Ca₂Cl was added to the incubation medium (the two columns on the right-hand side), and pictures were taken at the indicated time points. Each experiment was performed at least three times with identical results.

Figure 4. A intact Ca²⁺ binding domain is necessary for EGFP-cPLA₂ translocation in response to TR-PI(4,5)P₂. HEK cells transfected with the mutant EGFP-D43N-cPLA₂ were treated with TR-PI(4,5)P₂/histone in HBSS with 1.3 mM CaCl₂ and pictures were taken at the indicated time points by confocal microscopy. Each experiment was performed at least three times with identical results.



to potentially induce cPLA₂α membrane translocation (Gijón *et al.*, 2000a) (cf. Figure 6, A and B). Given these differences, we proceeded to determine the minimal Ca²⁺ requirements for TR-PI(4,5)P₂ to induce cPLA₂α translocation. To this end, cells were incubated in a Ca²⁺-free medium, exposed to either TR-PI(4,5)P₂ or ionomycin. Thereafter, different CaCl₂ concentrations were added to the incubation medium, and confocal microscopy images of the cPLA₂α movement were taken. [Ca²⁺]_i was monitored in Fluo-3-loaded cells in parallel under exact experimental conditions. Figure 7, A and B, show that TR-PI(4,5)P₂ promoted cPLA₂α membrane translocation at extracellular Ca²⁺ concentrations between 6 and 30 μM, which translate to 50–220 nM in terms of [Ca²⁺]_i. It is particularly striking that TR-PI(4,5)P₂-induced translocation of cPLA₂α begins to be detected in all the cells analyzed already at an extracellular Ca²⁺ concentration of 15 μM, which only induces minimal changes in [Ca²⁺]_i (~50 nM) (Figure 7, A and C). Note, in contrast, that ionomycin promotes visible translocation of cPLA₂α only at higher extracellular Ca²⁺ concentrations, ~60–150 μM, which raise

[Ca²⁺]_i up to 350–400 nM (Figure 7, B and D). These results provide strong evidence that TR-PI(4,5)P₂-induced cPLA₂α translocation occurs at substantially lower [Ca²⁺]_i levels than that triggered by ionomycin.

In vitro mutagenesis studies have identified a cluster of four Lys as the putative PtdInsP₂ binding site (Das and Cho, 2002; Six and Dennis, 2003). In the human enzyme, these are Lys⁴⁸⁸, Lys⁵⁴¹, Lys⁵⁴³, and Lys⁵⁴⁴. We constructed a mutant in which Lys⁴⁸⁸ was substituted by Glu and the three other Lys by Ala (EGFP-K488E/K541A/K543A/K544A-cPLA₂α; EGFP-4KE/A-cPLA₂α) (Das and Cho, 2002; Six and Dennis, 2003). When transfected into cells, the EGFP-4KE/A-cPLA₂α mutant actually translocated in response to TR-PI(4,5)P₂ (Figure 8A), although the degree of colocalization between mutant enzyme and phospholipid was considerably lower than that previously observed with the wild-type enzyme (Figure 8B). More importantly, cells transfected with the EGFP-4KE/A-cPLA₂α mutant did not mobilize AA in response to TR-PI(4,5)P₂, whereas cells transfected with the wild-type enzyme did it readily (Figure 9). These results demonstrate that the four-Lys cluster described by Das and Cho (2002) is indeed crucial for cPLA₂α to translocate to membranes in a functionally active form. That the EGFP-4KE/A-cPLA₂α mutant also translocates to membrane in response to PtdInsP₂ may suggest the existence of additional binding site(s) within the enzyme. However, because membrane binding of the EGFP-4KE/A-cPLA₂α mutant is not productive (i.e., no AA is released), it is probably of very limited biological significance. The behavior of the mutant EGFP-4KE/A-cPLA₂α clearly suggests that translocation of the enzyme to membrane does not necessarily lead to enhanced AA release.

It has previously been demonstrated that PtdInsP₂ does not increase the specific activity of the K541A/K543A/K544A mutant, as measured by an in vitro assay (Das and Cho, 2002; Six and Dennis, 2003). In our intact cell system, PtdInsP₂ promotes translocation of the construct EGFP-4KE/A-cPLA₂α to perinuclear membranes, but this is not accompanied by an increased AA release, which is in accordance with the aforementioned in vitro studies (Das and Cho, 2002; Six and Dennis, 2003). In these studies, an increase in the basal activity of the mutated enzyme compared with the wild-type enzyme was also shown. Our studies in intact cells failed to obtain reproducible evidence for such a phenomenon, as measured by basal AA release. It seems likely that in intact cells, the basal state of the cPLA₂α may be governed by multiple factors, some of which may not be apparent in an in vitro assay.

Our data, along with results from previous studies (Das and Cho, 2002; Six and Dennis, 2003), demonstrate that the positive charge generated by Lys⁴⁸⁸, Lys⁵⁴¹, Lys⁵⁴³, and Lys⁵⁴⁴ allows the cPLA₂α to properly bind to PtdInsP₂.

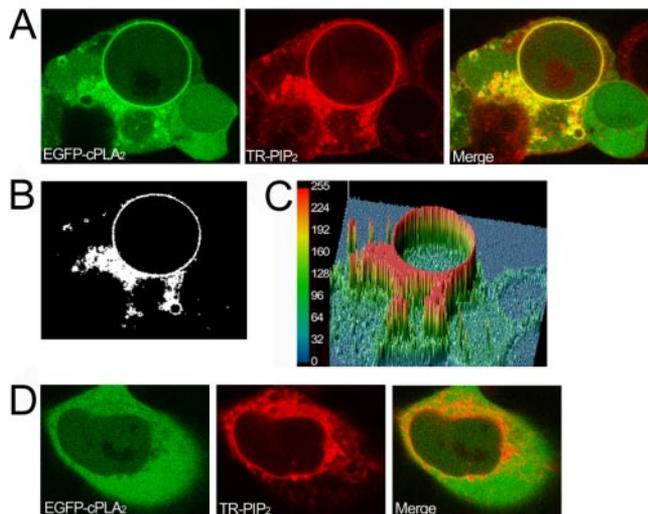


Figure 5. EGFP-cPLA₂ and TR-PI(4,5)P₂ colocalization analysis. HEK cells transfected with the construct EGFP-cPLA₂ were treated with TR-PI(4,5)P₂ for 20 min in the presence (A) or absence (D) of extracellular calcium, and pictures of green and red fluorescences were taken by confocal microscopy. The merge of both fluorescences is also shown. LaserPix software analysis of colocalization fluorescences in A is shown as a white mask (B) or as a colored three-dimensional representation, where red means maximum level of colocalization (255) and dark blue means the minimum (0) (C). Each experiment was performed at least three times with identical results.

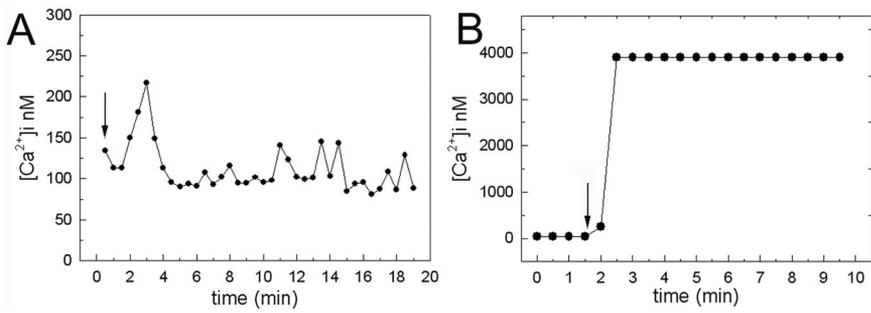


Figure 6. Intracellular Ca^{2+} levels in HEK cells. Intracellular measurements of Ca^{2+} were performed in Fluo-3-loaded HEK cells treated with TR-PI(4,5) P_2 /histone (A) or 5 μM ionomycin (B) in a buffer containing 1.3 μM CaCl_2 . Arrows indicate the time point at which the stimuli were added. Each data point represents the average of 20–40 cells. Fluorescence of Fluo-3 was detected by microscopy and recorded every 15–30 s. Each experiment was performed at least three times with identical results.

Mutation of these Lys leads to changes in the specific activity of the enzyme compared with the wild-type enzyme, as measured in an *in vitro* assay (Das and Cho, 2002; Six and Dennis, 2003). We have extended these data by showing decreased AA release in the mutants in response to PtdInsP_2 . The structure of those four Lys does not conform to the structure of well-known phosphoinositide binding

domains such as the plekstrin homology, the FYVE, or the Phox homology domains (Lemmon, 2003). It is likely that this cationic cluster belongs in a novel, yet-undefined phosphoinositide binding domain, as suggested previously (Das and Cho, 2002).

Based on the above-mentioned findings, it is intriguing to speculate with novel modes of $\text{cPLA}_2\alpha$ interaction with cel-

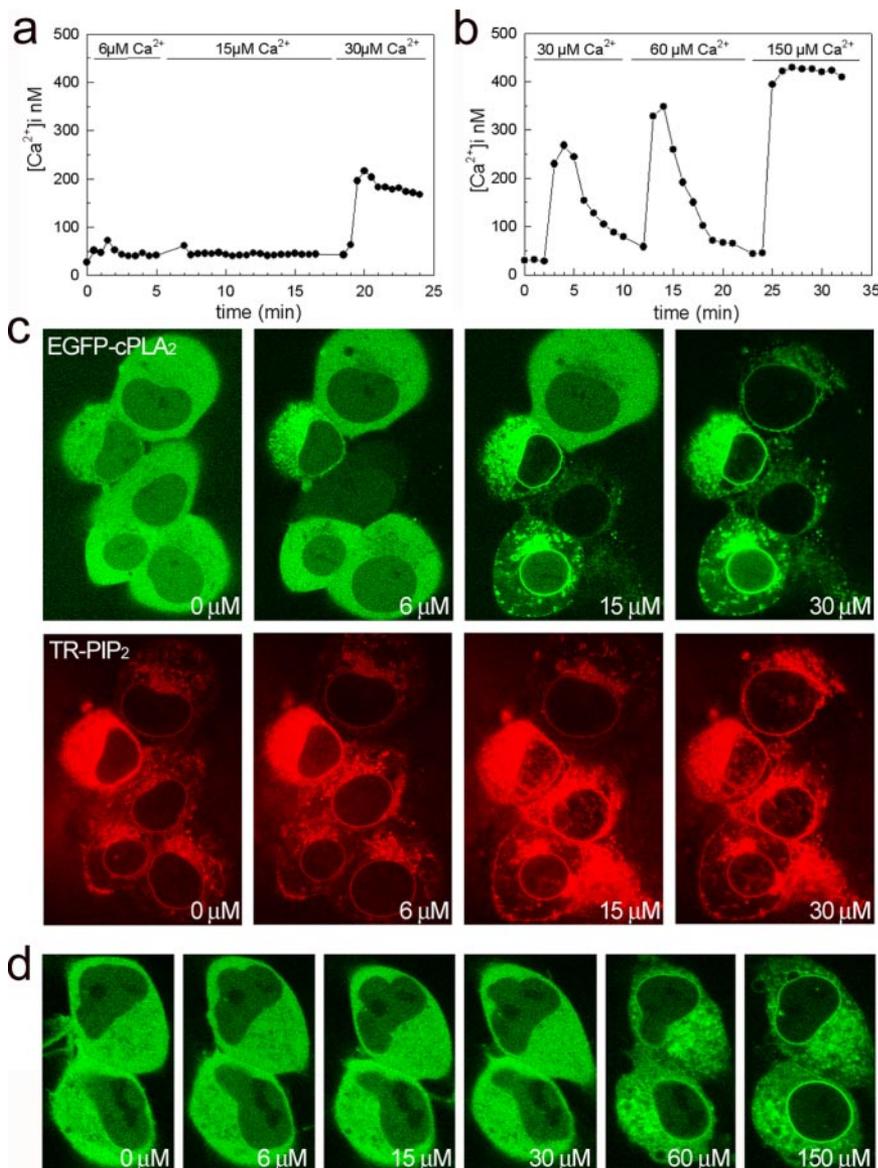


Figure 7. Intracellular Ca^{2+} requirements for TR-PI(4,5) P_2 -driven translocation of EGFP- cPLA_2 . Fluo-3-loaded HEK cells were first treated with TR-PI(4,5) P_2 /histone (A) or 5 μM ionomycin (B) in a buffer without Ca^{2+} and later, different CaCl_2 amounts were added as indicated. Fluorescence of Fluo-3 was detected by microscopy and recorded every 15–30 s. Each data point represents the average of 20–40 cells. Translocation of EGFP- cPLA_2 was analyzed by confocal microscopy under the same conditions indicated above for HEK cells [C, cells treated with TR-PI(4,5) P_2 /histone; D, cells treated with 5 μM ionomycin]. Extracellular concentration of CaCl_2 is indicated. Each experiment was performed at least three times with identical results.

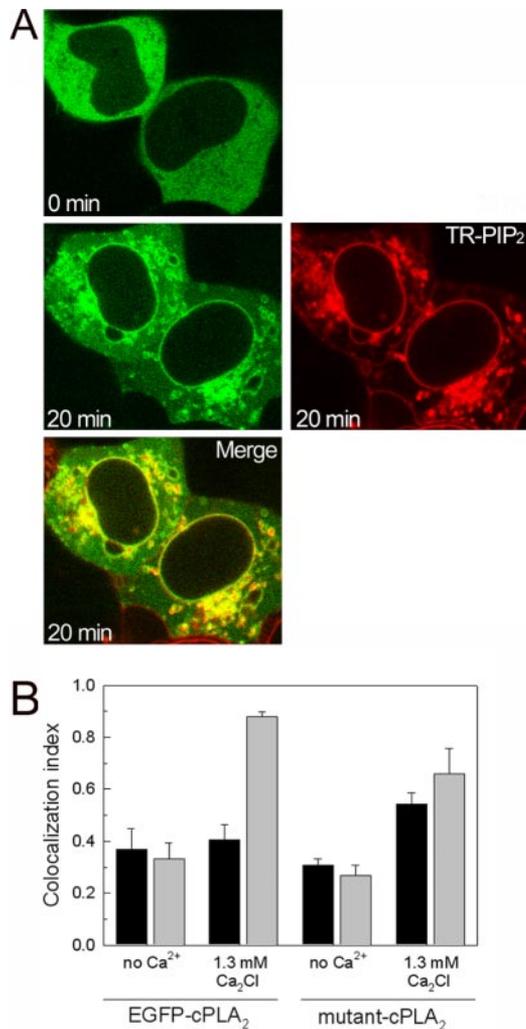


Figure 8. Analysis of the EGFP-4KE/A-cPLA₂α mutant. (A) HEK cells transfected with the mutant EGFP-4KE/A-cPLA₂α were treated with TR-PI(4,5)P₂/histone, and pictures were taken under the confocal microscope for the indicated time periods. (B) Colocalization analyses (LaserPix software) of the fluorescence from EGFP-4KE/A-cPLA₂α mutant and TR-PI(4,5)P₂ or EGFP-cPLA₂α and TR-PI(4,5)P₂ as a control, were performed in the presence of absence of extracellular calcium, as indicated. Colocalization index of red to green (black bars) or green to red (gray bars) is shown. Each data bar represents the average of 10–20 cells. Experiments were performed at least three times with identical results.

lular membranes leading to enhanced phospholipid hydrolysis. By using a methodology that preserves cellular integrity, i.e., shuttling fluorescent PtdInsP₂ into the cell by the use of lipophilic carriers, we have studied the interaction between PtdInsP₂ and cPLA₂α under a physiologically relevant setting, thus avoiding potential problems arising from the use of cellular fixation or permeabilization techniques. Our live cell studies clearly indicate that variations in the cellular PtdIns(4,5)P₂ may help regulate the physical location of cPLA₂α. Importantly however, for this regulatory role of PtdIns(4,5)P₂ to manifest, an intact C2 domain within the enzyme and a certain threshold level of intracellular calcium are required. The results suggest a sequential mode of cPLA₂α interaction with the membrane, where the enzyme first binds via the C2 domain in the presence of low Ca²⁺ levels. This is followed by binding to PtdIns(4,5)P₂ via

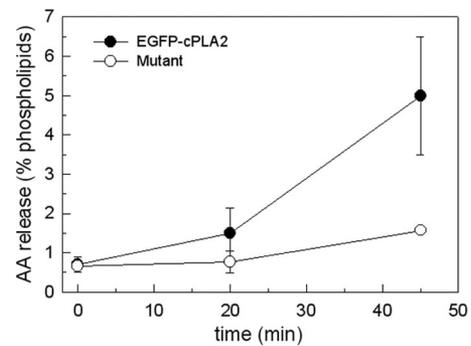


Figure 9. AA released by HEK cells transfected with the mutant EGFP-4KE/A-cPLA₂α. [³H]AA-labeled HEK cells transfected with the EGFP-cPLA₂α construct (closed circles) or the mutant EGFP-4KE/A-cPLA₂α (open circles) were treated with TR-PI(4,5)P₂/carrier and [³H]AA release was measured at the time indicated time points. Each experiment was performed at least three times with identical results.

the cationic cluster of four Lys, which positions the enzyme in the proper manner to effect phospholipid hydrolysis and AA release.

In this model of cPLA₂α activation by PtdIns(4,5)P₂, the threshold Ca²⁺ level required is far below that found in stimulated cells and resembles more that of resting cells, i.e., ~50 nM. This is in contrast with studies *in vitro*, which reflected no Ca²⁺ requirement for cPLA₂α binding to and hydrolysis of phospholipids in mixed micelles (Six and Dennis, 2003). This difference highlights the very complex nature of the interaction of cPLA₂α with a biological membrane in a live cell context. cPLA₂α has been shown to physically interact with ceramide 1-phosphate (Subramanian *et al.*, 2005) and a number of proteins, including vimentin (Nakatani *et al.*, 2000) and a splice variant of Tip60 (Sheridan *et al.*, 2001). Thus, it is likely that in cells, numerous entities in addition to PtdInsP₂ may interact with cPLA₂α and modulate its membrane binding properties.

It is important to note that the above-mentioned scenario does not exclude the possibility that PtdInsP₂ binding may occur first, and this in turn augments the calcium-dependent lipid binding of the C2 domain. Such a sequence of events has received some experimental support by the recent work of Subramanian *et al.* (2005) in which ceramide 1-phosphate was found to increase cPLA₂α activity by interacting with the C2 domain, thereby lowering the calcium requirement for translocation.

Our findings provide a mechanistic insight into the intracellular signaling triggered by stimuli such as bacterial lipopolysaccharide, UV light, phorbol esters, or okadaic acid, which do not mobilize Ca²⁺ from internal stores, yet they all activate the cPLA₂α-mediated release of AA (Shinohara *et al.*, 1999; Gijón *et al.*, 2000b). It is likely that the increased synthesis of PtdInsP₂ that occurs during cellular stimulation by these agonists (Taylor *et al.*, 1984; Balsinde *et al.*, 2000), together with the low nanomolar levels of [Ca²⁺]_i, provides the signals to anchor cPLA₂α to perinuclear membranes. Two subfamilies of kinases, termed type I and type II, are involved in the biosynthesis of PtdIns(4,5)P₂ during cell activation. Type I consists of PtdIns(4)P 5-kinases, whereas type II consists of PtdIns(5)P 4-kinases. Members of both subfamilies have been localized in the perinuclear region of cells (Doughman *et al.*, 2003). On the basis of our findings, we propose that PtdIns(4,5)P₂ formed by phosphatidylinositol monophosphate kinases at the perinuclear envelope

may help recruit cPLA₂α to this compartment at low [Ca²⁺]_i levels. Future studies should expand on the mechanisms governing PtdIns(4,5)P₂ accumulation in perinuclear membranes and their overall role in cPLA₂α-mediated AA signaling.

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