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Participation of prostaglandin D_2 in the mobilization of the nuclear-localized CTP:phosphocholine cytidylyltransferase alpha in renal epithelial cells

Nicolás O. Favale [a], Lucila G. Pescio [a], Bruno J. Santacreu [a], María G. Márquez [b], Norma B. Sterin-Speziale [c,*]

[a] Department of Biological Sciences, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, IQUIFIB-CONICET, Ciudad Autónoma de Buenos Aires C1113AAD, Argentina
[b] Instituto de Investigaciones en Ciencias de la Salud Humana (IICSHUM), Universidad Nacional de La Rioja, La Rioja, Argentina
[c] LANAIS-PROEM-IQUIFIB-CONICET, Ciudad Autónoma de Buenos Aires C1113AAD, Argentina

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ABSTRACT

Phosphatidylcholine (PC) is the main constituent of mammalian cell membranes. Consequently, preservation of membrane PC content and composition — PC homeostasis — is crucial to maintain cellular life. PC biosynthetic pathway is generally controlled by CTP:phosphocholine cytidylyltransferase (CCT), which is considered the rate-limiting enzyme. CCTα is an amphitropic protein, whose enzymatic activity is commonly associated with endoplasmic reticulum redistribution. However, most of the enzyme is located inside the nuclei. Here, we demonstrate that CCTα is the most abundant isoform in renal collecting duct cells, and its redistribution is dependent on endogenous prostaglandins. Previously we have demonstrated that PC synthesis was inhibited by indomethacin (Indo) treatment, and this effect was reverted by exogenous PGD_2. In this work we found that Indo induced CCTα distribution into intranuclear Lamin A/C foci. Exogenous PGD_2 reverted this effect by inducing CCTα redistribution to nuclear envelope, suggesting that PGD_2 maintains PC synthesis by CCTα mobilization. Interestingly, we found that the effect of PGD_2 was dependent on ERK1/2 activation. In conclusion, our previous observations and the present results lead us to suggest that papillary cells possess the ability to maintain their structural integrity through the synthesis of their own survival molecule, PGD_2, by modulating CCTα intracellular location.

1. Introduction

Phosphatidylcholine (PC) is the main constituent of mammalian cell membranes. Consequently, preservation of membrane PC content and composition — PC homeostasis — is crucial to maintain cellular life. In renal papillae, PC comprises around 45% of the total phospholipid content. Although this zone of the kidney exhibits the lowest phospholipid mass, it possesses the highest PC biosynthetic activity [1]. Works from our laboratory have contributed to elucidating how such an active metabolic process is maintained and regulated in the renal tissue, demonstrating the main role of prostaglandins (PGs) as regulators of phospholipid biosynthesis [2,3]. Papillary PC synthesis is dependent on endogenous PGs, in particular prostaglandin D_2 (PGD_2), which operates by modulating the activities of PC biosynthetic enzymes [4].

PC is predominantly synthesized in mammalian cells and tissue by the CDP-choline pathway [5]. The CDP-choline pathway involves three steps: choline is first phosphorylated by choline kinase; thereafter, phosphocholine condenses with CTP to form CDP-choline in a reaction catalyzed by CTP:phosphocholine cytidylyltransferase (CCT). CDP-choline, in turn, donates the phosphocholine moiety to diacylglycerol (DAG) to form PC by mediation of DAG:CDP-choline transferase (CPT). The biosynthetic pathway is generally controlled by CCT, which is considered the rate-limiting enzyme [6]. CCT is an amphitropic protein, whose enzymatic activity is regulated by its association with membranes [7,8]. It contains no binding pockets for lipid monomers, but responds to changes in membrane physical properties, such as changes in surface density or lipid composition [7]. Four isoforms of CCT, CCTα, CCTβ1, CCT β2 and CCT β3, have been characterized. The beta isoforms are splicing variants of the same gene, whereas the alpha isoform is encoded by a separate gene [9]. The four isoforms are similar in their catalytic and membrane binding domains and are all regulated by lipids [9]. A careful evaluation of CCT localization in several cell types has demonstrated that CCTα is found associated with membranes in the cytoplasm, but due to its N-terminal domain, which contains a nuclear localization signal [10–12], it could be located inside the nucleus.

Nuclear localization of CCTα was first reported by Wang et al. [13], and it has been considered as a diffuse pool of the enzyme available to translocate to the membrane to become active. We further showed that nucleoplasmic CCTα adopts a high degree of organization depending on the physiological status of the cell [14]. The localization of CCTα in mammalian cells is still a matter of controversy. Northwood et al. [15] proposed the translocation of CCTα from the nucleus to the cytoplasm

* Corresponding author.
E-mail address: speziale@ffyb.uba.ar (N.B. Sterin-Speziale).

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as a mechanism for CCTα activation that accompanies its oscillation during cell cycle. By contrast, Ridsdale and Post reported that in pulmonary tissue cells, most CCTα is located outside the nucleus in association with glycogen pools [16] and is independent of the cell cycle [17]. On the other hand, Delong et al. have reported constitutive localization of CCTα in the nucleus with no evidences of translocation outside the nucleus and demonstrated that nuclear CCTα localization is a cell type- and cell cycle-independent event in mammalian cells [12]. In CHO, Hela and liver cells, nucleoplasmic CCTα translocates to the nuclear envelope for activation in response to several stimuli, including the presence of fatty acids [18], PC degradation [19] and isoprenoids [20]. More recently, translocation of nuclear CCTα into intranuclear tubules of the nucleoplasmic reticulum has been found to promote cellular proliferation, triggered by fatty acids [21].

It is now accepted that the mammalian nucleus is a highly structured and dynamic compartment that contains numerous morphologically well-defined structural units, including the nucleolus and several "nuclear bodies", such as the Cajal body and the promyelocytic leukemia body [22,23]. Additionally, a large volume of nuclear space is occupied by a compartment commonly referred to as "nuclear speckles". Nuclear speckles were first described as RNA splicing factor compartments [24], but an increasing number of proteins, such as Rb and PI kinases, have been reported to be included in these nuclear speckles [25,26]. The biological function of nuclear speckles is not completely understood, but it has been proposed that they are involved in the regulation of transcriptional events [27] and myoblast differentiation [25].

In a previous work from our lab, we have demonstrated that when MDCK cells — a cell line derived from dog renal collecting duct cells — are subjected to external hypertonicity, CCTα translocates to intranuclear Lamin A/C foci, as a strategy to regulate the enzyme activity [14]. Based on these results and our early report about the endogenous PGD_2 dependence on renal papillary PC synthesis, we decided to evaluate whether the regulatory effect of PGs on PC biosynthesis is due to changes in the intracellular distribution of CCTα.

2. Materials and methods

2.1. Materials

Hybond-P membranes were purchased from GE Healthcare (Fairfield, USA). X-ray film for autoradiography was from Eastman Kodak Co. (Rochester, USA). PGD_2, indomethacin (Indo), and metabolic inhibitors were purchased from Sigma-Aldrich (St. Louis, USA). Polyclonal CCTα and CCTβ antibodies were kindly provided by Dr. Suzanne Jackowski (St. Jude Children's Research Hospital). All other reagents and chemicals were of analytical grade (Merck or Mallinckrodt) and purchased from local commercial suppliers.

2.2. Tissue treatment and subcellular membrane preparation

Male Wistar rats (body weight: 250–300 g) were sacrificed by decapitation, and both kidneys removed and kept in an ice-cold Krebs-Ringer buffer, pH 7.4, containing 5.5 mM glucose gassed with 95% O_2/5% CO_2. Each kidney was cut in halves through the pelvis along its longitudinal axis and the papillae (whitish inner medulla) were isolated by scissors and scalpel dissection, and sliced by using Staddie-Riggs microtome. Tissue slices (100 mg wet weight) were collected in 200 μl of ice-cold Tris–HCl buffer, pH 7.4, containing 5.5 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$ (TEG solution) in the presence or absence of 0.5 μM Indometacin (Indo) to avoid PGs synthesis and incubated at 37 °C. After 60 min, papillary samples were stimulated with 0.1 μM PGD_2 for 1, 5 and 15 min, and then rapidly processed for membrane fractionation or microscopy studies (see below). In another set of experiments, after 60 min incubation, samples were stimulated with 0.1 μM PGD_2 for 1,5 and 15 min either in the absence or in the presence of 1 μM U0126. After treatment, papillary slices were immediately homogenized in 10 vol of a solution 0.25 M sucrose containing 25 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF, 1 μg/ml leupetin, 1 μg/ml aprotinin and 1 mM $NaVO_4$, and successively centrifuged at 860 ×*g* for 10 min, 8000 ×*g* for 20 min, and 105,000 ×*g* for 60 min. The 105,000 ×*g* pellet was used as microsomal fraction (MF). To obtain enriched nuclei fraction (ENF), the pellet obtained from the first sedimentation was resuspended in 3.8 vol of a 2.4 M sucrose solution containing 25 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$ and 2 mM EGTA, 1 mM PMSF, 1 μg/ml leupetin, 1 μg/ml aprotinin and 1 mM $NaVO_4$, and centrifuged at 50,000 ×*g* for 60 min; the resulting pellet was used as nuclear fraction [31,32]. The purity of nuclei was assessed by microscopy and by the measurement of glucose 6P-phosphatase and 5′ nucleotidase activities, to evaluate endoplasmic reticulum (ER) and plasma membrane contamination, respectively [33]. These assays showed that 2.4 M sucrose pellets were highly enriched in nuclei. The activity of 5′ nucleosidase was 40 ± 13 and 1080 ± 198 nmol of inorganic phosphorus/mg protein in 60 min in the nuclear fraction and microsomal membranes, respectively. Glucose 6P phosphatase activity was 210 ± 106 and 1840 ± 215 nmol of inorganic phosphorus/mg protein in 60 min in nuclei and microsomes, respectively, corresponding to the fact that the outer nuclear membrane is contiguous with the ER. The different fractions were resuspended in the corresponding reaction buffer and aliquots were separated to evaluate protein content and kept at –85 °C until their use for western blot.

2.3. Western blot assays

Sample aliquots containing 100 μg of protein from each subcellular compartment were incubated for 5 min at 100 °C with Laemmli-sample buffer, resolved by electrophoresis in a 12.5% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes. After blotting, membranes were treated with 5% non-fat milk in TBS-Tween 20, incubated with primary antibodies at 4 °C overnight. The antibodies used were: 1:500 of rabbit polyclonal antibody against CCTα or CCTβ, 1:2000 mouse anti-lamin A/C (Millipore), 1:5000 mouse anti-actin (Millipore) or 1:3000 rabbit anti-Na/K ATPase (Santa Cruz). Primary interaction was evidenced using the enhanced chemiluminescence kit ECL (GE). The intensity of each band was estimated by optical densitometry with Gel-Pro Analyzer 3.1.

2.4. Immunohistochemistry

Tissue slices were obtained as described above and fixed in formaldehyde 10% in phosphate buffer saline (PBS) for 2 h at room temperature. Fixed tissue was embedded in paraffin and mounted slice was obtained. Paraffin was cleaned by xylene and absolute ethanol washes. Then tissue was hydrated by PBS washes, blocking with blocking buffer (PBS containing 3% BSA and 3% goat pre-immune serum) for 1 h at room temperature in a humid chamber. The tissue sections were incubated with primary antibody against CCTα or CCTβ at 1:100 diluted in blocking buffer overnight at 4 °C in a humid chamber. After PBS washing, primary interactions were evidenced by using secondary biotinylated antibody (Vector) and avidin-biotin-peroxidase complex (ABC-HRP complex, DakoCytomation) and revealed by DAB-peroxidase (Sigma).

2.5. Primary culture of collecting ducts

Primary culture of papillary collecting duct cells was performed by means of the procedure by Stokes et al. [17]. Briefly, renal papillae were minced to 1–2 mm^3 pieces and incubated at 37 °C in sterile TBS containing 0.1% (*w*/*v*) collagenase under 95% O_2/5% CO_2 for 40 min. From collagenase digestion, a "crude-pellet", containing most papillary cell types and tubular elements, was obtained by centrifugation at 175 g for 10 min. The pellet was washed twice and resuspended in Dulbecco's modified Eagle's medium (DMEM/F-12), 10% (*v*/*v*) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and an

"enriched papillary collecting duct pellet" was obtained by centrifuging at 60 g for 1 min. Pellets were resuspended in DMEM/F12 and aliquots placed on sterile dry-glass coverslips. After 96 h, the cells grown on coverslips were incubated at 37 °C in Tris–HCl buffer, pH 7.4, containing 5.5 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$ (TEG solution) with 1 μM Indometacin (Indo). After 60 min, samples were stimulated with 0.1 μM PGD_2 for 1 and 15 min, and then rapidly processed for immunofluorescence. In another set of experiments, after 60 min incubation, samples were stimulated with 0.1 μM PGD_2 for 1 or 15 min either in the absence or in the presence of 1 μM U0126.

2.6. Fluorescence microscopy

Cells were plated on coverslips and treated as described above. Cells were washed with PBS, and fixed with 4% (*w*/*v*) paraformaldehyde at 25 °C for 20 min, and permeabilized with 0.1% (*v*/*v*) Triton X-100 at 25 °C for 20 min. After washing with PBS, cells were incubated with 3% BSA and 3% normal goat serum in PBS at 25 °C for 60 min, followed by incubation at 25 °C for 90 min with different primary antibodies. The antibodies used were: 1:100 rabbit anti-CCTα and 1:100 mouse anti-Lamin A/C (Millipore). Primary interactions were detected by using a 1:250 F(ab´)2 fragment of goat anti-rabbit immunoglobulin G (IgG) conjugated with AlexaFluor-488 and a 1:250 F(ab´)2 fragment of goat anti-mouse IgG conjugated with AlexaFluor-594 (Jackson ImmunoResearch). *Dolichos biflorus* agglutinin (DBA) conjugated with TRITC has been used to confirm the identity of collecting duct cells [28].

Nuclei and ER were labeled with TOTO3 (Molecular Probes) and Concanavalin A conjugated with AlexaFluor-647 (Molecular Probes), respectively. The coverslips were then mounted onto microscope glass slides with Vectashield Mounting Media (Vector Lab) and stored at 4 °C until analysis.

2.7. Imaging and data processing

Images of confocal immunofluorescence were obtained by an Olympus FV300 confocal microscope (model BX61) equipped with Ar and He–Ne lasers, and oil immersion 60× numerical aperture 1.4. Images were taken with the acquisition software FluoView version 3.3. Optical sections of 0.25 μm for Z-plane reconstruction were obtained. For image analysis and reconstruction, ImageJ and Image-Pro plus version 4.5 were used.

3. Results

3.1. CCTα is expressed in renal papilla collecting duct cells

Two isoforms of CCT (CCTα and CCTβ) can be expressed in mammalian tissues. To evaluate the CCT isoform expressed in renal papillae, immunohistochemistry assays using anti-CCTα or anti-CCTβ antibodies were performed. Renal papillary specimens showed CCTα-positive signal (Fig. 1A-a) but no signal for CCTβ (Fig. 1A-b). Interestingly, the CCTα signal was concentrated in collecting ducts (CD) (Fig. 1A-a white arrow) showing low signal in the rest of the renal papilla (Fig. 1A-a white arrowhead). These results can be visualized in the magnified image of the transversal section of CD (Fig. 1A-a'), where the positive signal is restricted to the CD. To discard false positives, a negative control (avoiding the primary antibody) was performed (Fig. 1A-c). To obtain additional evidence, CCTα immunofluorescence was also performed and CCTα-positive signal appeared restricted to the CD (Fig. 1B white arrow), with low signal in the rest of the renal papillary structures (white arrowhead).

To obtain a biochemical approach, we performed a western blot analysis of CCTα and CCTβ in papillary homogenate and demonstrated that CCTα was expressed in the renal papillary tissue while CCTβ signal was almost undetectable (Fig. 1C). To corroborate that CCTβ antibody functions a western blot analysis was performed in liver homogenate (data not shown).

To corroborate the selectivity of CCTα expression in CD cells, CD cells were further isolated (as described in materials and methods) and a western blot analysis of CCTα was performed in both non-CD and CD-enriched fractions (Fig. 1D). A CCTα-positive band was observed in the CD-enriched fraction, but was absent in non-CD fraction, thus confirming the low signal obtained either in the immunohistochemistry as well as in the immunofluorescence assays. It is important to note that

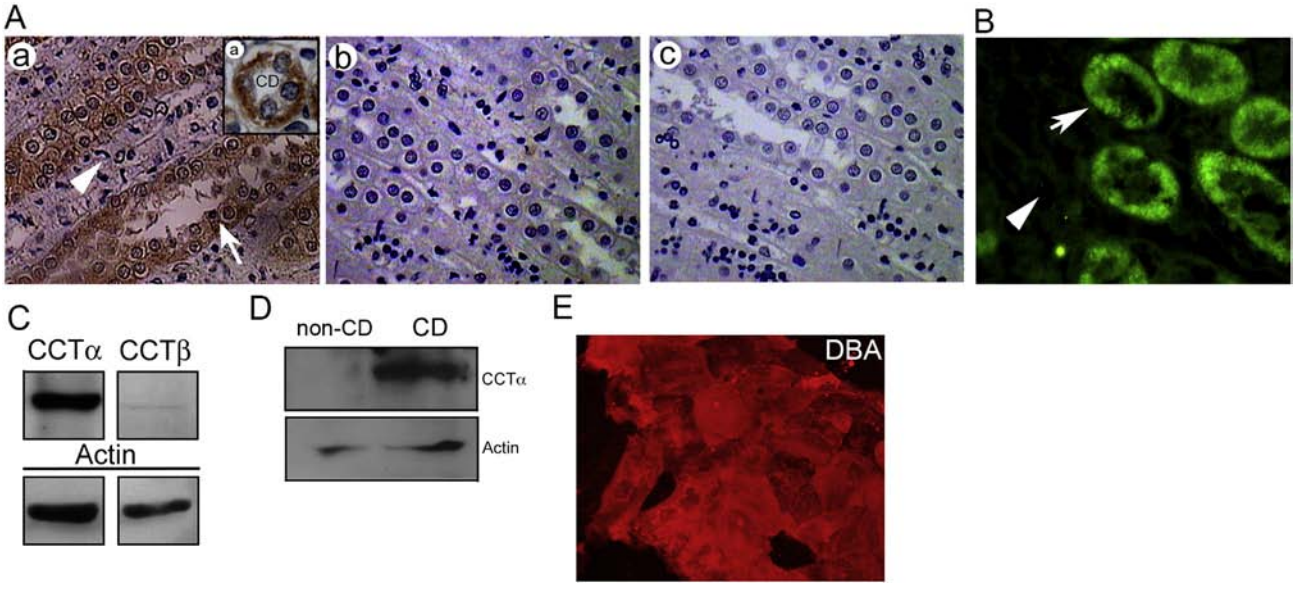

Fig. 1. *CCTα is expressed in renal papilla collecting duct cells.* (A) Immunihistochemistry of CCTα in rat renal papillary tissue (a) Immunohistochemistry shows CCTα-positive signal in renal papilla collecting duct cells (white arrow) and no signal in interstitial cells (white arrowhead). (a´) CCTα-positive signal is observed in a transversal section of collecting duct (CD). (b) No positive signal for CCTβ is observed. (c) A negative control was performed avoiding the primary antibody. No positive signal is observed. Hematoxylin stain was used to recognize tissue morphology. (B) Immunofluorescence shows CCTα-positive signal in renal papilla collecting duct cells (white arrow) and no signal in interstitial cells (white arrowhead). (C) Western blot analysis shows CCTα expression in renal papilla homogenate. (D) Western blot analysis of CCTα in collecting duct (CD) cells and non-CD cells. Actin was used as loading control. (E) To confirm the identity of CD cells, we used DBA a known marker of adult collecting cells.

non-CD fraction presented CCTα expression but was considerable lower than CD-enriched fraction, and a higher amount of protein charge was necessary to be detected (data not shown).

To confirm that the identity of collecting duct cells we used DBA a known marker of adult collecting cells (Fig. 1E).

3.2. PGD_2 induces CCTα intracellular redistribution

We have previously demonstrated that indomethacin (Indo), the most potent inhibitor of PG synthesis, produces a decrease in CCT activity and PC synthesis [29]. The reversion of the Indo inhibitory effect perfectly correlated with PGD_2 addition in a time-dependent manner [30]. In the present study, we evaluated whether the PGD_2-induced increase of PC synthesis was due to changes in CCTα amount. To this end, we performed a western blot analysis of papillary homogenates obtained from Indo pre-treated and stimulated tissue with 0.1 μM PGD_2 for 1, 5 or 15 min (Fig. 2A). The western blot showed that PGD_2 induced no changes in CCTα levels, suggesting that the recovery of PC synthesis exerted by PGD_2 was independent of the changes in CCTα protein level.

Since it is known that CCTα could be localized in different subcellular compartments and its subcellular localization is related with the enzyme activity [6,8,31], we evaluated whether PGD_2 was able to change CCTα intracellular distribution. For this purpose, we isolated the microsomal fraction (MF), the enriched nuclei fraction (ENF) and the cytosolic fraction (CF) from renal papillary tissue stimulated with 0.1 μM PGD_2 for 1, 5 and 15 min. To avoid the effect of endogenous-synthesized PGD_2, tissue was pretreated with 1 μM Indo. A western blot analysis was performed for the different subcellular fractions. The signal obtained in tissue treated with Indo without PGD_2 stimulation was considered as control (Fig. 2 black bars). PGD_2 induced a transient increase in the amount of CCTα in MF and ENF (Fig. 2B white bars). After 1 min of stimulation, approximately 100% increase over the control value was observed in ENF, decreasing after 5 min. In MF, CCTα remained increased after 5 min. After 15 min, the amount of CCTα associated with ENF as well as that associated with MF returned to control values. Interestingly, PGD_2 induced a decrease in cytosolic CCTα after 1 and 5 min with a recovery of its pool after 15 min. These results suggest that PGD_2 modulates CCTα intracellular distribution, probably due to a mobilization from the cytosolic pool to the particulate (ENF and MF) pool.

3.3. Signaling pathway involved in PGD_2–stimulated CCTα redistribution

We have previously demonstrated that the PGD_2 modulation of PC biosynthesis is dependent on the ERK1/2-mediated pathway and that the effect is blocked by U0126 (an ERK1/2 inhibitor) [30]. To evaluate ERK1/2 mediates the PGD_2 effect on CCTα redistribution, we studied the CCTα amount in the different cellular fractions in the presence of U0126. For this purpose, renal papillary slices were stimulated with PGD_2 in the presence of U0126. The inhibitor abolished the PGD_2-induced CCTα increase in MF and ENF (Fig. 2B, C and D gray bars) and also blocked the enzyme decrease in the soluble fraction at 1 min of stimulation. A significant decrease in the enzyme was observed after 15 min in MF, concomitantly with a significant increase in the soluble fraction (CF).

The results suggest that PGD_2 induces a mobilization of CCTα from the soluble to the particulate intracellular fraction in an ERK1/2-dependent manner.

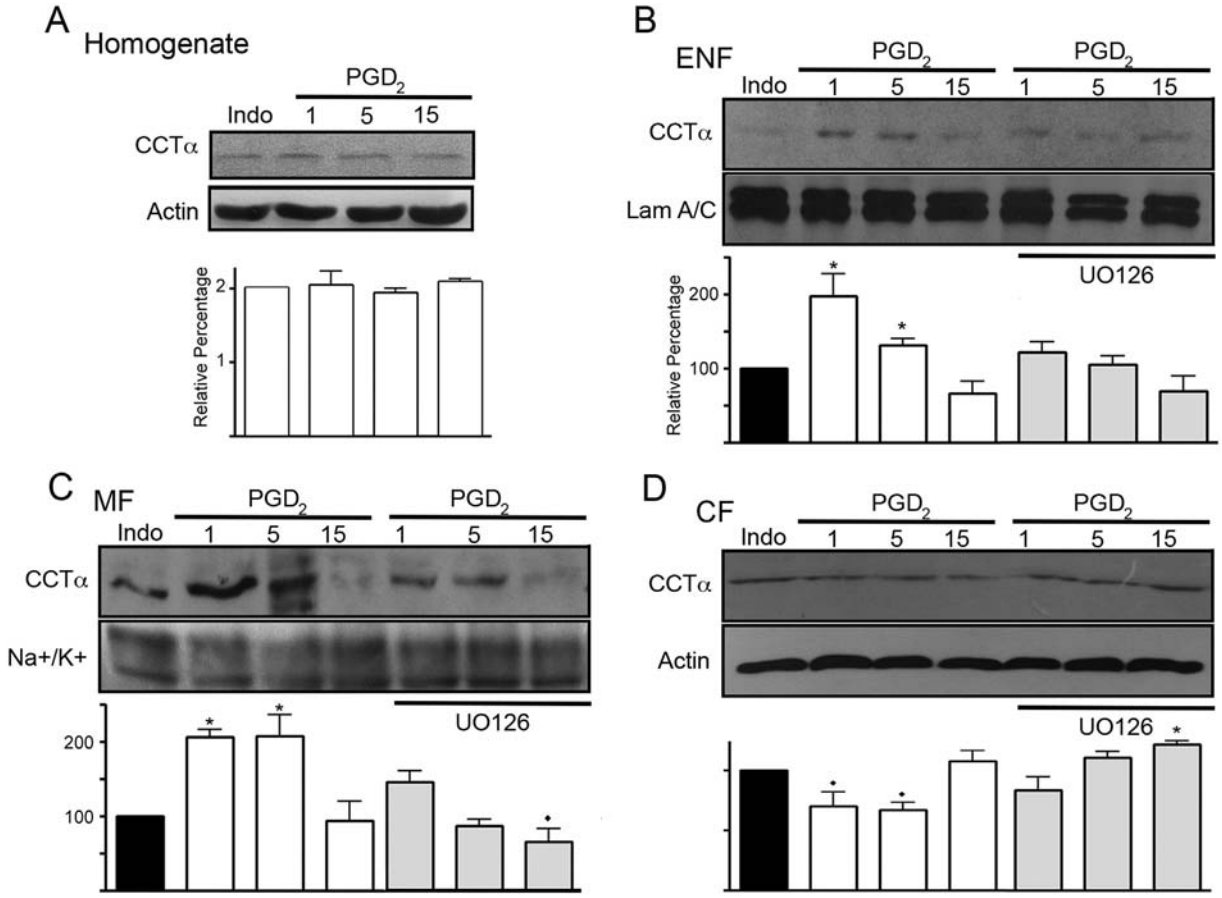

Fig. 2. *Prostaglandins maintain CCTα intracellular distribution.* (A) PGD_2 effect on CCTα protein levels. Western blot assay in papillary homogenates treated with Indo and later stimulated with 0.1 μM PGD_2 for 1, 5 and 15 min. PGD_2 and ERK1/2 effects on CCTα subcellular distribution: (B) enriched nuclear fraction (ENF), (C) microsomal fraction (MF) and (D) cytosolic fraction (CF). Different proteins were used as loading control: Lamin A/C in the ENF, Na/K ATPase in the MF and actin in the CF.

3.4. Intracellular CCTα distribution depends on PGD_2

To obtain more precise evidence on the CCTα intracellular distribution, we performed indirect immunofluorescence on primary cultured CD cells. To verify the identity of the intracellular structure, we used Concanavalin A (ConA) and anti-Lamin A/C (Lam A/C) as markers of the ER and nucleus, respectively. ConA showed positive label in the cytoplasm and was negative in the cell center (Fig. 3A-a white "*"). The negative zone for ConA corresponded to the nucleus, which was positive for nuclear lamina labeling by the Lamin A/C antibody (Fig. 3A-b). In control-cultured cells, CCTα appeared mostly cytoplasmically distributed with perinuclear accumulation (Fig. 3A-c, white arrowhead). Intranuclear positive foci were also observed. The merged images show that CCTα accumulation corresponds to the nuclear envelope (NE) and to the ER (Fig. 3A-d white arrowhead and 3A-e).

To evaluate the effect of endogenous PGs in the intracellular distribution of CCTα, primary cultured cells were treated with Indo 1 μM for 60 min before the performance of the immunofluorescence microscopy. Indo treatment caused a CCTα intracellular redistribution, showing a more diffuse cytoplasmic distribution with dissipation of the perinuclear accumulation (Fig. 3B-c) and inclusion of CCTα in intranuclear Lamin A/C-positive foci. These results suggest that endogenous PGs are necessary for the maintenance of the intracellular CCTα distribution.

We further studied the involvement of PGD_2 in Indo-induced CCTα translocation. After 1 min of PGD_2 stimulation (Fig. 3C), CCTα started to accumulate in the nuclear periphery (Fig. 3C-c white arrowhead) as seen in the merged image. After 15 min (Fig. 3D and Fig 1 supplementary), CCTα concentrated in the nuclear envelope, acquiring a clear perinuclear distribution (Fig. 3D-c white arrowhead), with dissipation from the cytoplasmic ER. These results suggest that PGD_2 induced a progressive intracellular redistribution of CCTα.

Thereafter, we carried out a line profile analysis as a way to obtain a semiquantitative relationship between the relative spatial distribution of Lamin A/C, ConA, CCTα and the degree of colocalization. Representative magnified images show the prompts where the line profile analyses were performed (Fig. 4A-a, B-b, C-a and D-b). The graphs represent a relative intensity of fluorescence in each point (pixels) analyzed by distance (microns). In all the experimental conditions (Fig. 4A, B, C and D), we observed that the maximum level of Lamin A/C (circles) intensity of fluorescence corresponded to nuclear lamina. On the other hand, ConA (squares) presented lower fluorescence intensity in the nuclear matrix and higher intensity in the cytoplasm, consistent with ER localization. The CCTα signal in control cells (Fig. 4A-triangles) followed the ConA profile of distribution, reflecting that in control conditions CCTα was mostly distributed in the cytoplasm, specifically in the ER. When the cells were treated with Indo (Fig. 4B), we observed that the enzyme showed an increase in the relative fluorescence in the nucleoplasm,

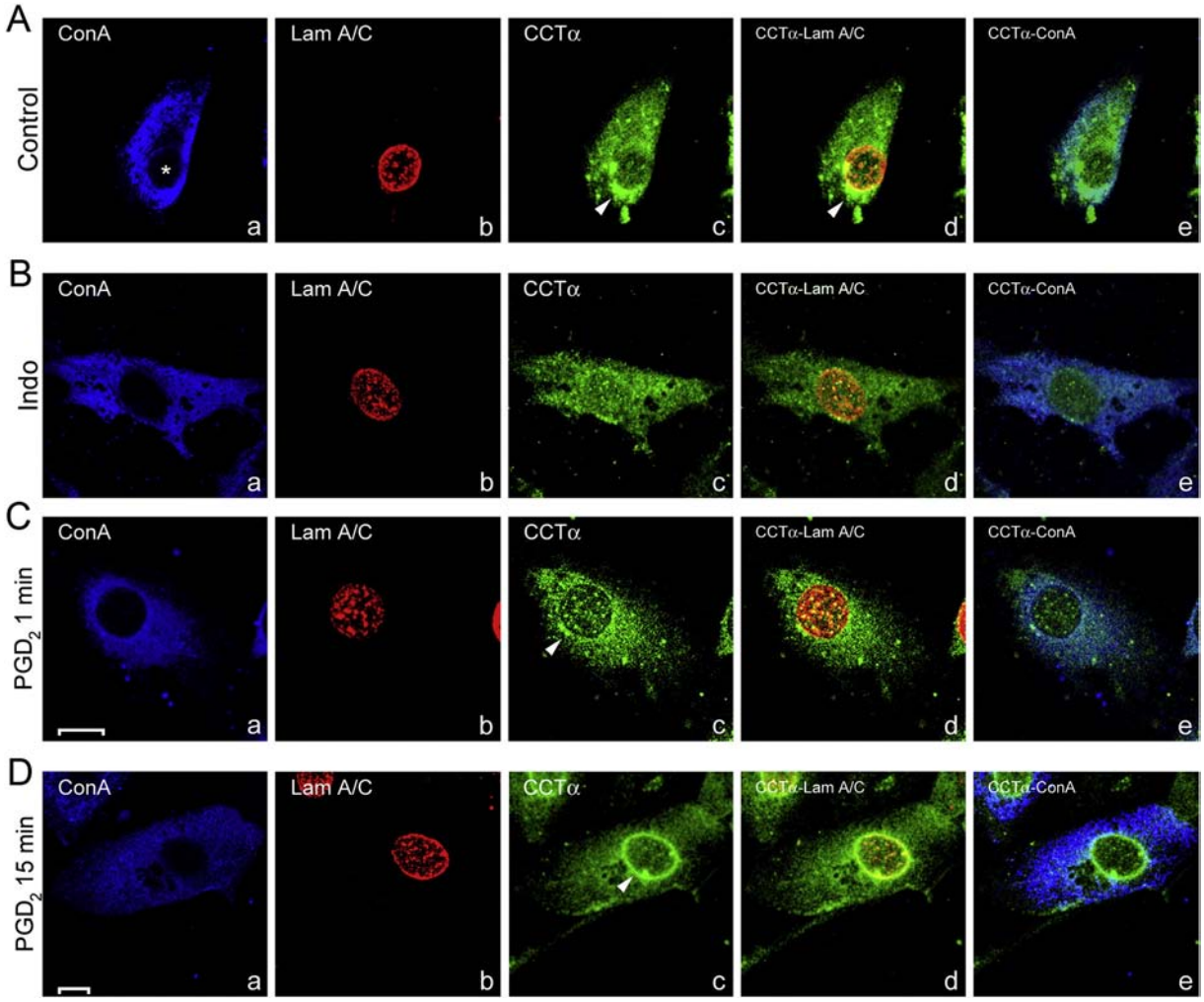

Fig. 3. *PGD_2 induces CCTα intracellular translocation.* Indirect immunofluorescence of primary cultured collecting duct cells: (a) Concanavalin A (ConA), (b) anti-Lamin A/C (Lam A/C) as markers of ER and nucleus respectively, and (c) anti-CCTα to detect CCTα location. Merged images were obtained (d and e). (A) Control cells, (B) Indo-treated cells, (C) Indo-treated cells stimulated with PGD_2 for 1 min, (D) Indo-treated cells stimulated with PGD_2 for 15 min.

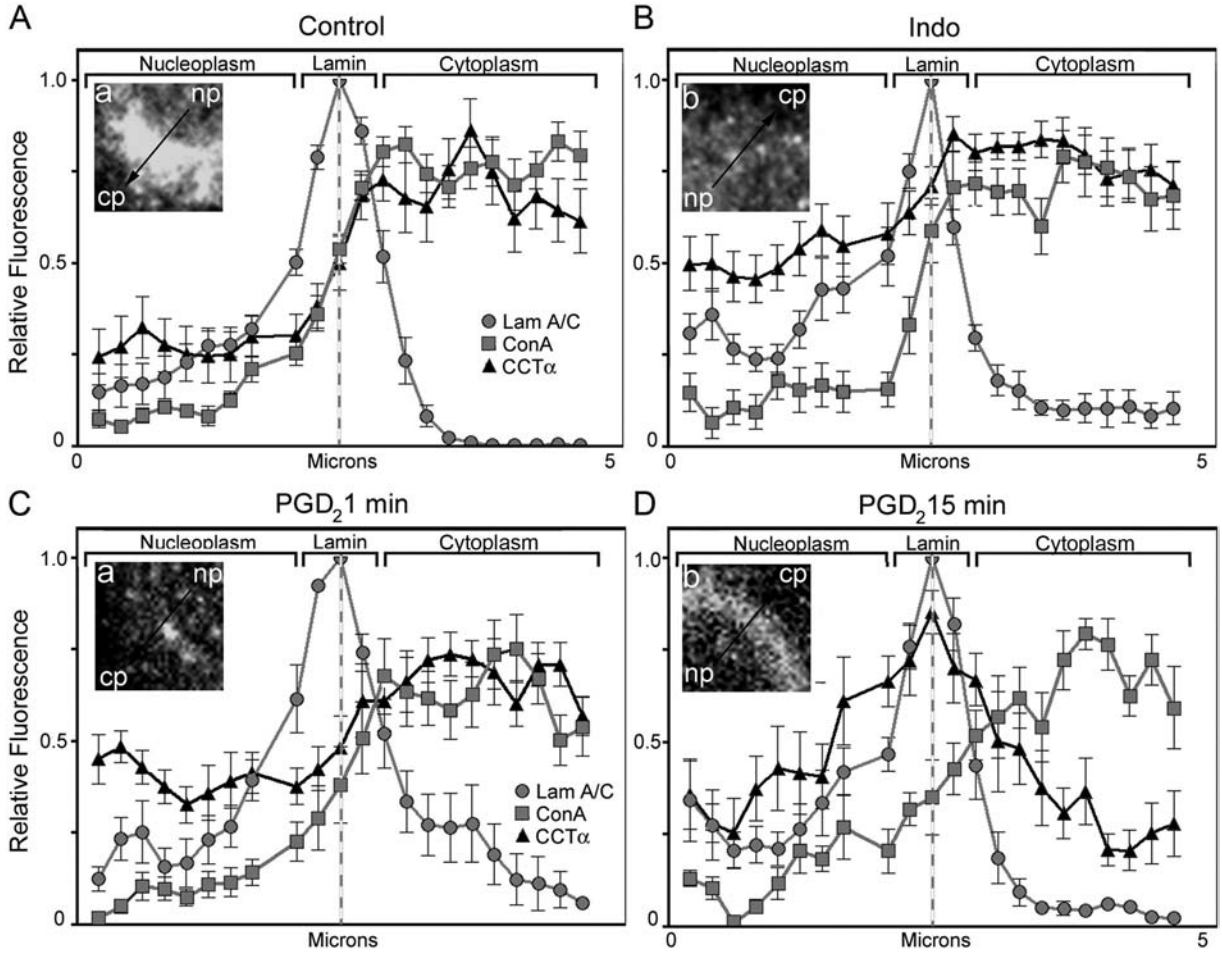

Fig. 4. *Line profile analysis of fluorescence distribution.* A line profile analysis was performed, as a way to obtain a semiquantitative relationship between the relative spatial distribution of Lamin A/C (circle), ConA (square), CCTα (triangle) and the degree of colocalization. Representative magnified images show the prompts where the line profile analyses were performed (a, b, c and d). The graphs represent the relative intensity of fluorescence in each point (pixels) analyzed by distance (microns). (A) Control cells, (B) Indo-treated cells, (C) Indo-treated cells stimulated with PGD_2 for 1 min, (D) Indo-treated cells stimulated with PGD_2 for 15 min.

being almost equally distributed in the nucleoplasm and cytoplasm. When Indo pretreated cells were stimulated with PGD_2 for 1 min, a relative intensity of fluorescence of CCTα modestly decreased in the nucleoplasm (Fig. 4C), and started its discontinuous perinuclear accumulation (Fig. 3C-c white arrowhead). After 15 min, the CCTα accumulation in the perinuclear region became evident. Indeed, CCTα distribution almost paralleled the Lamin A/C profile, thus suggesting that PGD_2 mobilized CCTα to the nuclear envelope (Fig. 4D).

3.5. Intracellular CCTα mobilization by PGD_2 depends on ERK1/2 activation

To evaluate the involvement of ERK1/2 in the PGD_2-induced CCTα redistribution, we cultured the cells in the presence of U0126 as described in materials and methods, and performed an immunofluorescence assay. At 1 min stimulation (Fig. 5A), CCTα remained almost equally distributed in the nucleoplasm and the cytoplasm. After 15 min of stimulation, CCTα appeared concentrated in the nuclear periphery (Fig. 5B). The semiquantitative analysis of the spatial fluorescence distribution showed an increase in CCTα signal in the nucleus that colocalized with Lamin A/C, suggesting that PGD_2 induces CCTα redistribution from the cytoplasmic ER to the nuclear envelope (NE) (Fig. 5C and D). Nevertheless, the effect was significantly lower when the cells were stimulated with PGD_2 in the absence of U0126 (Fig 4D). These results suggest that the CCTα intracellular redistribution by PGD_2 is dependent on ERK1/2 activation.

3.6. Primary collecting duct cells present intranuclear lamin A/C foci

To obtain precise information about the nuclear CCTα distribution and mobilization, we performed confocal immunofluorescence microscopy, focused on the nuclear confocal plane. We used Lamin A/C as a marker of nuclear structures. We have previously demonstrated that in an epithelial renal cell line (MDCK cells) CCTα is distributed in intranuclear Lamin A/C foci. Moreover, the enzyme mobilization to Lamin A/C foci occurred under a differentiating stimulus like hypertonic stress medium [14]. Figs. 3 and 5 show that in fully differentiated primary cultured renal papillary CD cells, the nucleus presents nucleoplasmic Lamin A/C signal. To distinguish the nucleoplasmic foci from the intranuclear projections of nuclear membrane, an optical sectioning was carried out at 0.25-μm intervals (Fig. 6A a-h). Such procedures allowed us to evidence that the Lamin A/C-containing structures were located throughout the nucleoplasm. Moreover, foci were observed all over the sequential focal sections, evidencing their intranuclear distribution (Fig. 6A white arrow a-c, blue arrow d-h and green arrow e-g).

It has been described that intranuclear Lamin A/C could be associate with the nucleoplasmic reticulum (NR) [32]. To rule out this possibility we obtained immunofluorescence confocal sections using Alexa Fluor conjugated-Con A (ConA), a lectin currently used as an NR tubule marker. ConA was increased in signal and showed in gray scale to improve NR detection. As showed in Fig. 6B (a-h) high ConA signal was observed in

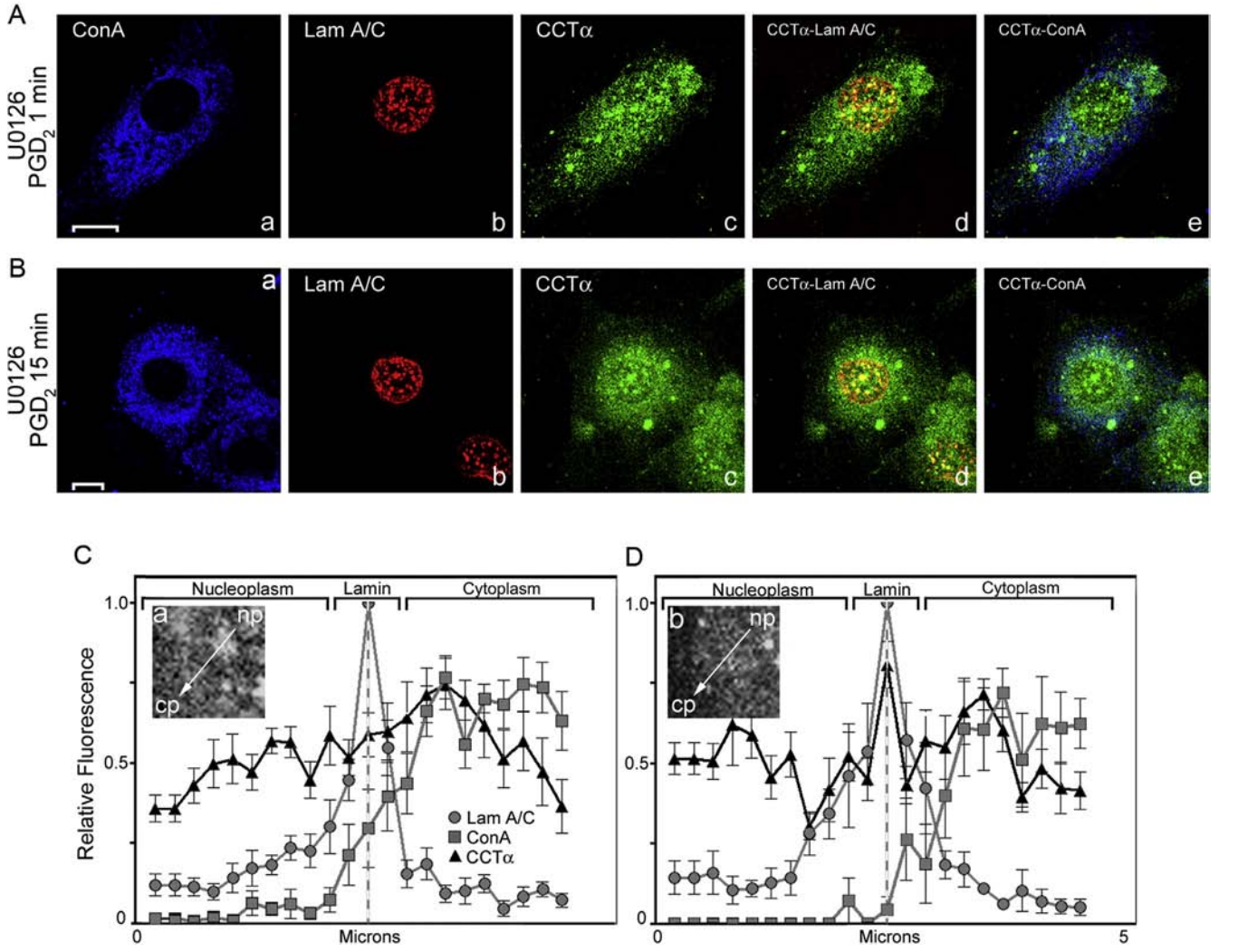

Fig. 5. *CCTα intracellular translocation induced by PGD2 is dependent on ERK1/2 activation.* Indirect immunofluorescence of primary cultured collecting duct cells treated with U0126 before PGD_2 stimulation: (a) ConA and (b) anti-Lam A/C as markers of the ER and nucleus respectively, and (c) anti-CCTα to detect CCTα location. Merged images were obtained (d and e). (A) Indo- and U0126-treated cells stimulated with PGD_2 for 1 min. (B) Indo- and U0126-treated cells stimulated with PGD_2 for 15 min. (C) Fluorescence line profile distribution of Indo- and U0126-treated cells stimulated with PGD_2 for 1 min. (D) Fluorescence line profile distribution of Indo- and U0126-treated cells stimulated with PGD_2 for 15 min.

the nuclear envelope and its proximity decreasing inside the nuclear matrix.

When primary cultured CD cells were double-labeled with mouse anti-Lamin A/C and ConA, Lamin A/C showed intranuclear localization, with a foci pattern of distribution (Fig. 6C-b). By contrast, the ConA signal was distributed in the perinuclear region staining the ER, but the fluorescence signal was very low in the nuclear matrix. To obtain more detailed information, we carried out a z-plane projection by the reconstruction from series of 0.25-μm optical sections and cross section images (Fig. 6C-c- lines 1, 2, 3 and 4). The z-plane projections obtained from lines 1, 2, 3 and 4 of the distribution of Lamin A/C and ConA are shown in Fig. 6C. The merged image confirms the nucleoplasmic localization of Lamin A/C (1, 2, 3 and 4) without ConA colocalization. Fig. 6D represent a middle plane of the nuclear region stained with ConA (a) Lamin A/C (b), and the merge image (c). The magnified image of an individual Lamin A/C nuclear focus (white square a´, b´ and c´), shows that ConA was not included in the foci but were present in the surrounding (white arrowhead).

In order to determine if intranuclear CCTα colocalize with the Lamin A/C nuclear foci, a z-plane reconstruction of Indo-treated cells was performed (Fig. 6E). We observed that Lamin A/C foci colocalized with CCTα, suggesting the co-distribution of both proteins.

3.7. Inhibition of endogenous PGs induces CCTα intranuclear lamin A/C foci redistribution

Since it has been found that nuclear CCTα associates with Lamin A/C when its membrane binding is promoted [32], and we have previously demonstrated that in MDCK cells CCTα associates with Lamin A/C intranuclear speckles [14], we further evaluated the intranuclear distribution of Lamin A/C and CCTα by confocal microscopy.

In control cells, positive Lamin A/C signal was observed in the perinuclear region, suggesting NE localization, and also in intranuclear foci in control cells (Fig. 7A-a). CCTα presented a diffuse intranuclear signal but appeared concentrated in the nuclear periphery (Fig. 7A-b). The merged image (Fig. 7A-c) shows perinuclear but not intranuclear points of CCTα-Lamin A/C colocalization, better observed in binarized images (Fig. 7A-d), where colocalization between both fluorophores is seen in white. In this image only a perinuclear distribution was observed, confirming no intranuclear CCTα location. To obtain more details about CCTα intranuclear distribution, merged images were magnified. The zone of magnification was delimited by a white square (Fig. 7A-c). The magnified images show that nuclear Lamin A/C foci did not colocalize with CCTα (Fig. 7A-e).

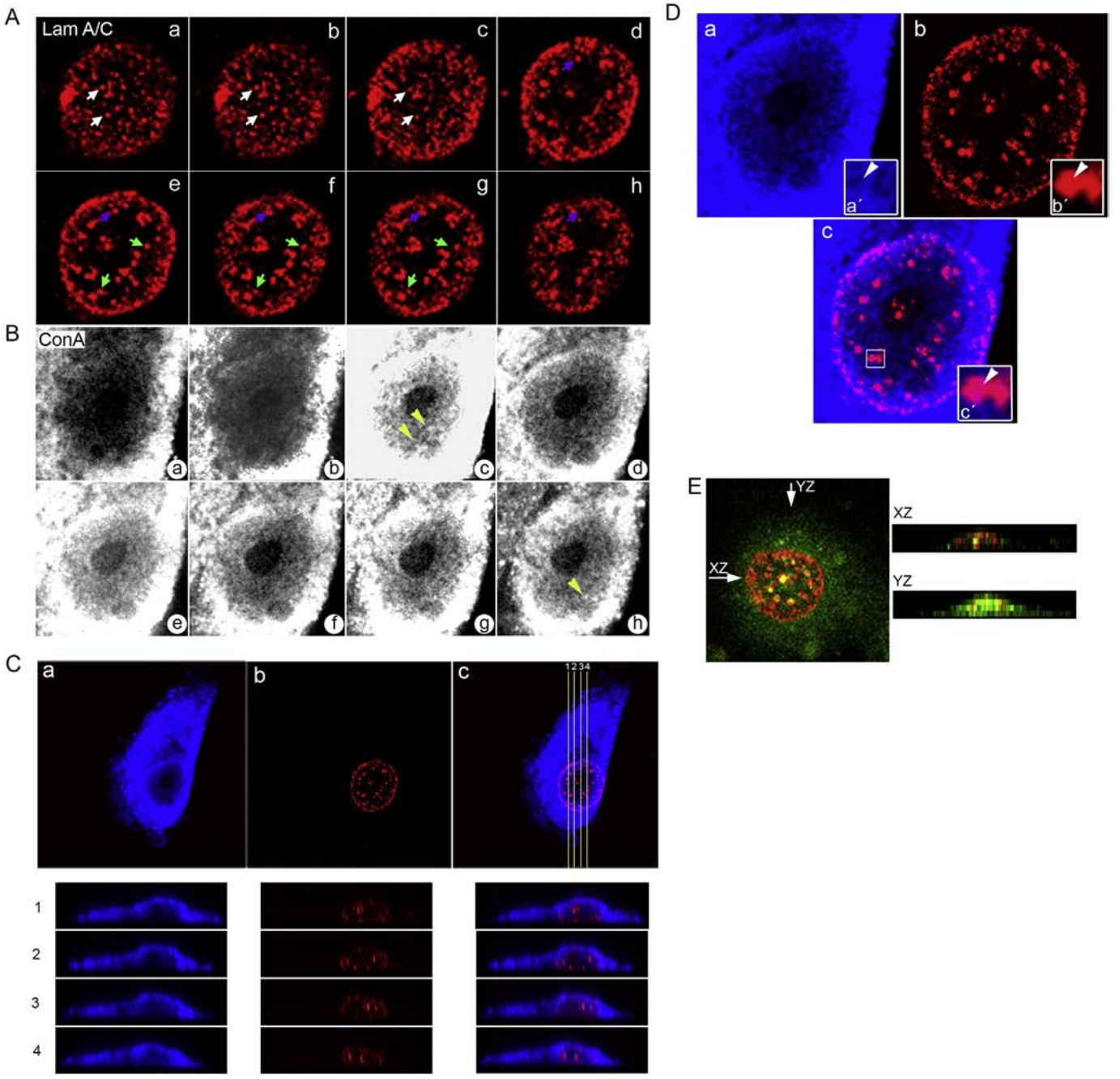

Fig. 6. *Intranuclear Lamin A/C distribution.* (A and B) To distinguish the nucleoplasmic foci from the intranuclear projections of nuclear membrane, an optical sectioning was carried out at 0.25-μm intervals (a–h). (C) z-plane projection by the reconstruction from a series of 0.25-μm optical sections and cross sections images were obtained (lines 1, 2, 3 and 4). (D) Middle plane of the nuclear region stained with ConA (a) Lamin A/C (b), and the merge image (c). Magnified image of an individual Lamin A/C nuclear foci were obtained (white square a´, b´ and c´). (E) XZ and YZ plane reconstructions of CCTα (green) and Lamin A/C (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

When cultured cells were treated with Indo (to inhibit endogenous PG synthesis), no change in Lamin A/C distribution was observed (Fig. 7A-f). However, CCTα lost its perinuclear distribution (Fig. 7A-g) and some intranuclear CCTα-positive foci that colocalized with Lamin A/C were observed (Fig. 7A-g). These observations were confirmed in the segmentation (Fig. 7A-i) and in the magnified image (Fig. 7A-j white arrows). These results suggest that when endogenous PG synthesis was inhibited by Indo treatment, CCTα dissipated from the NE and appeared distributed into the nucleoplasm.

We have previously demonstrated that PGD_2 was able to reconstitute PC synthesis. Thus, we decided to evaluate whether this PC synthesis involves intranuclear CCTα redistribution. For this purpose, primary cultured CD cells were treated with Indo and then stimulated with 0.1 μM PGD_2 for 1 or 15 min. After 1 min of PGD_2 stimulus, we observed a decrease in CCTα foci and some accumulation of the enzyme in the perinuclear region (Fig. 7B-b). This effect was more evident after 15 min of PGD_2 stimulation, where we observed a decrease in the number of CCTα intranuclear foci that colocalized with Lamin A/C with an accumulation of CCTα in the NE (Fig. 7B g-i).

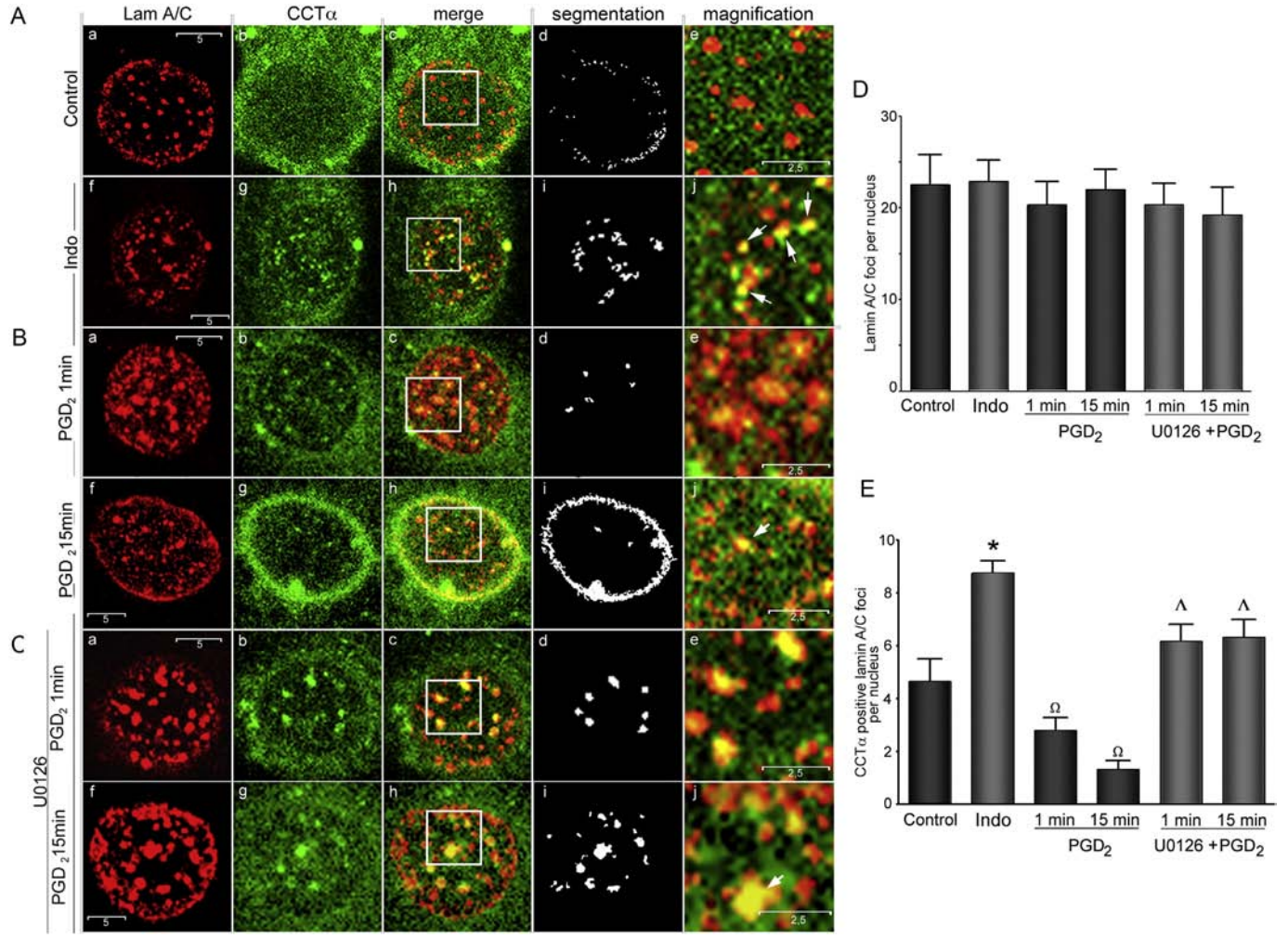

Fig. 7. Effect of PGD_2 and ERK1/2 in the intranuclear CCTα-Lamin A/C foci distribution. Evaluation of the intranuclear distribution of Lamin A/C (a) and CCTα (b) by confocal microscopy in control cells and treated with Indo (A) and cells treated with Indo and stimulated with PGD_2 for 1 and 15 min (B). Merged (c) and segmentation (d) images were obtained. To obtain more details about CCTα intranuclear distribution, from the zone delimited by a white square, merged images were magnified (e). (C) Cells treated with Indo and U0126, and stimulated with PGD_2 for 1 and 15 min. (D) Quantitative analysis of the number of Lamin A/C foci per nuclei and (E) CCTα-Lamin A/C foci per nuclei.

3.8. PGD_2 induces CCTα mobilization out of lamin A/C foci in a mechanism dependent on ERK1/2

To evaluate whether or not ERK1/2 mediates PGD_2 effect on CCTα intranuclear foci mobilization, we stimulated primary cultured CD cells with PGD_2 in the presence of 1 μM U0126 and performed immunofluorescence.

In the presence of the ERK1/2 inhibitor, PGD_2 was not able to induce CCTα mobilization out from Lamin A/C foci at 1 min or at 15 min of stimulation (Fig. 7C). Instead of mobilizing, it seems that CCTα-Lamin A/C foci increased in size, reflecting CCTα accumulation within the foci and avoiding its presence in the NE (Fig. 7C d, e, i and j).

To obtain a quantitative analysis, the number of intranuclear Lamin A/C foci and CCTα-Lamin A/C foci was determined. Neither Indo nor PGD_2 stimulation modified the number of Lamin A/C foci (Fig. 7D), thus suggesting that in these experimental conditions Lamin A/C foci were independent of endogenous PG synthesis. However, a significant increase in the number of CCTα-Lamin A/C foci was observed when cells were treated with Indo (Fig. 7E), and this effect was reversed after PGD_2 stimulation. These results show a direct relationship between PGs, particularly PGD_2, and CCTα intranuclear localization. When the analysis was performed in the presence of MAPK inhibitor the number of Lamin A/C foci was not modified (Fig. 7D). However, U0126 blocked the PGD_2-induced redistribution of CCTα outside the foci as well as NE accumulation (Fig. 7E). These results suggest that PGD_2 intranuclear redistribution is dependent on ERK1/2 activation.

4. Discussion

The present work offers a new piece of evidence about the role of PGD_2 on renal papillary CCTα mobilization and demonstrates that PGD_2 evokes an ERK-dependent intracellular CCTα distribution in a mechanism that involves the intranuclear and cytoplasmic redistribution of the enzyme.

In previous works from our lab, we have demonstrated that papillary PC synthesis is maintained by endogenous PGs [1,3,33], being PGD_2 the one that keeps the best correlation, thus assigning an important new role for such PG in the kidney. We have also reported that PGD_2 exerts its effect on PC synthesis by modulating the activities of PC biosynthetic enzymes [4,29]. Further, we demonstrated that PGD_2 modulation of PC synthesis is dependent on ERK1/2-MAPK activation [30]. Since it has been widely reported that CCTα is the rate-limiting enzyme on PC biosynthesis and that its translocation from soluble to membrane compartments (amphytrophism property) is its major mechanism of activation [8], in the present study we evaluated the effect of endogenous PGs, particularly PGD_2, on CCTα expression and intracellular distribution. The intracellular localization of CCTα in mammalian cells is still a matter of controversy. Northwood et al. [15] proposed that the translocation

of CCTα from the nucleus to the cytoplasm is a mechanism for CCTα activation. By contrast, Ridsdale and Post reported that in pulmonary tissue cells, most CCTα is located outside the nucleus in association with glycogen pools [17]. On the other hand, Delong et al. reported constitutive CCTα in the nucleus, with no evidences of translocation outside the nucleus [12]. In a previous work from our lab, we showed that in MDCK cells, CCTα is exclusively located in the nucleus and contained in the intranuclear domain of Lamin A/C foci (speckles), and proposed that CCTα inclusion into speckles is a strategy to assure enzyme availability for PC biosynthesis when it is physiologically demanded [14]. In the present study, we showed that both in renal papillary slices and in primary cultures of renal papillary collecting duct cells, CCTα is nuclear and cytoplasmic located. At this point, we could suggest that the collecting duct cell line (MDCK) probably lost the capacity to cytoplasmically locate the CCTα. Based on our observations and those of others, we can conclude that CCTα intracellular distribution is dependent on the cellular type, and differences between cell lines and primary cultured cells are evident.

After demonstrating that CCTα but not CCTβ is the isoform expressed in adult rat renal collecting duct cells, and that neither Indo nor PGD_2 change the endogenous level of CCTα, we studied the CCTα intracellullar distribution at two levels. First, we analyzed the cytoplasmic-nuclear distribution and secondly we focused the analysis at the nuclear level. Although it is commonly accepted that the ER is the main site of PC synthesis, our results show that under conditions of inhibition of its synthesis, as occurs by treatment with Indo, CCTα remained located in the ER and when PC synthesis was restored (at 15 min of PGD_2 stimulation) CCTα distributed in the NE. Also in control conditions, when PC synthesis is active, colocalization of the enzyme with the NE marker (Lamina A/C) is evident. These observations led us to suggest that the CCTα located in the nuclear envelope but not that located in the ER could be the most implicated in the maintenance of PC biosynthesis.

It is interesting to note that the inhibition of endogenous PG synthesis evoked by Indo produced changes in the intracellular pattern of CCTα distribution, mainly causing its release from the NE to be accumulated in nuclear Lamin A/C foci, thus suggesting that the absence of endogenous PGs induces the entry of CCTα to an inactive compartment. When cells were stimulated with PGD_2 the level of nuclear CCTα was similar to that of controls, and after 15 min of PGD_2-stimulation nuclear CCTα was almost totally located in the nuclear periphery.

Neither the inhibition of the endogenous synthesis of PGs nor the PGD_2 stimulation produced changes in the endogenous level of CCTα as demonstrated in the Western blot analysis, thus highlighting the concept that the particulate association of CCTα plays a central role in the modulation of its activity.

We have previously demonstrated that PGD_2 increases PC biosynthesis by an ERK1/2-dependent mechanism (Fig. 2 supplementary). To evaluate whether the CCTα redistribution was ERK1/2-dependent, we performed a western blot analysis and indirect immunofluorescence in the presence of an inhibitor of ERK1/2 activation. In this condition we observed that CCTα was unable to be redistributed from nuclear Lamin A/C foci to the NE. Our previous observation about PC synthesis and this present result on CCTα location may reinforce the concept that nuclear foci are reservoirs of inactive CCTα and that to become active the enzyme must be located in the nuclear membrane.

In renal papillary cells, preservation of membrane homeostasis is extremely important since this tissue is immersed in aggressive environments [34], and no active cell turnover is present in adult tissue. We have also demonstrated that active turnover of membrane phospholipids is the strategy that renal papillary collecting duct cells use to preserve their homeostasis [33].

In conclusion, our previous observations and the present results led us to suggest that papillary cells possess the ability to maintain their structural integrity through the synthesis of their own survival molecule, PGD_2, which by modulating CCTα intracellular location can safeguard the membrane homeostasis by the regulation of PC synthesis, and constitutes “per se” a physiological protective mechanism for collecting duct cells that are continuously exposed to aggressive environments such as the high interstitial osmolality.

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Transparency documents

The Transparency documents associated with this article can be found, in online version.

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