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ORIGINAL ARTICLE

# Membrane Lipid Composition Plays a Central Role in the Maintenance of Epithelial Cell Adhesion to the Extracellular Matrix

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**Abstract** Focal contacts (FC) are membrane-associated multi-protein complexes that mediate cell-extracellular matrix (ECM) adhesion. FC complexes are inserted in detergent-resistant membrane microdomains enriched in phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$); however, the influence of membrane lipid composition in the preservation of FC structures has not been extensively addressed. In the present work, we studied the contribution of membrane lipids to the preservation of renal epithelial cell adhesion structures. We biochemically characterized the lipid composition of membrane-containing FC complexes. By using cholesterol and PtdIns(4,5)$P_2$ affecting agents, we demonstrated that such agents did not affect any particular type of lipid but induced the formation of new FC-containing domains of completely different lipid composition. By using both biochemical approaches and fluorescence microscopy we demonstrated that phospholipid composition plays an essential role in the in vivo maintenance of FC structures involved in cell-ECM adhesion.

**Keywords** Membrane lipids · Focal contacts · Collecting duct cells · Cell-extracellular matrix adhesion

**Abbreviations**

| | |
|---|---|
| DRM | Detergent-resistant membrane domains |
| ECM | Extracellular matrix |
| FC | Focal contacts |
| IB | Immunoblotting |
| IP | Immunoprecipitation |
| CD | Methyl-$\beta$-cyclodextrin |

## Introduction

Tissue organization depends on structures that enable cells to attach to the extracellular matrix (ECM). Focal contacts (FC), the most stable points of cell tethering to the ECM, are multi-protein complexes that reach the outside of the cell from the cytoplasm through the cell membrane and contact the actin-cytoskeleton inside the cell with ECM proteins [1, 2]. FC assembly occurs by the binding of the integrin-extracellular domain to the ECM proteins followed by the interaction of the $\beta$-integrin cytoplasmic domain with talin, which, in turn, binds vinculin [3–5]. The acidic phospholipid, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$), acts as a regulator of both actin dynamics and FC turnover [6], and many cytoskeleton proteins involved in cell adhesion bind PtdIns(4,5)$P_2$ in order to be

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either activated or inactivated [7]. It is accepted that both vinculin activation and integrin binding to talin are dependent on plasma membrane local availability of PtdIns(4,5)$P_2$ [8].

Both talin and vinculin need to be inserted into the phospholipid bilayer to form FC plaques. Earlier in vitro studies have demonstrated that the insertion of either vinculin or talin into the lipid bilayer depends on the bilayer phospholipid composition [9]. On the other hand, it has been reported that many of the molecular components that regulate cell-ECM adhesion are associated with cholesterol-enriched detergent-resistant membrane microdomains (DRM), which are also enriched in PtdIns(4,5)$P_2$ [10]. It is considered that the specific interactions with phosphoinositides could serve to recruit cytoskeletal proteins to particular membrane domains, in which specific lipids are thought to be accumulated [10].

In spite of the relevance that membrane lipids appear to have for FC assembly, there are no previous reports on the membrane lipid composition where FC are located in vivo or on how changes in local membrane composition contribute to FC maintenance.

In the present study, we first performed the biochemical characterization of the membrane domains where FC complexes are located in renal papillary collecting duct cells. Thereafter, by combining biochemical and immunofluorescence studies, we obtained experimental evidence showing how specific changes in the membrane lipid composition in vivo are a sufficient condition to affect the maintenance of FC, thus demonstrating that membrane lipid composition plays a central role in the maintenance of cell-ECM adhesion.

## Materials and Methods

Silicagel plates for thin layer chromatography (TLC) were from Merck. X-ray film was obtained from the Eastman Kodak Co. Neomycin, LiCl, methyl-$\beta$-cyclodextrin, Collagenase Type II, Fast 3,3′diaminobenzidine tablets and monoclonal antibodies against vinculin and talin and phalloidin-FITC were from the Sigma Chemical Co (St. Louis, MO, USA). Biotinylated anti PtdIns(4,5)$P_2$ was from Echelon Biosciences Inc. (Lake City, UT), FITC-conjugated goat F(ab)$_2$ to mouse IgG was from Jackson ImmunoResearch Inc. (West Grove, PA) and ABC complex/HRP Kit was from Dako Lab (Glostrup, Denmark). Protein A/G PLUS Agarose was obtained from Santa Cruz Biotechnology Inc (Burlingame, CA). All culture reagents were from Gibco (Gibco, Invitrogen, USA). All other reagents and chemicals were of analytical grade (Sigma, Merck or Mallinckrodt) and purchased from local commercial suppliers.

### Animals and Tissue Preparation

Adult male Wistar rats were sacrificed by cervical dislocation and both kidneys removed. Renal papillae were isolated by scalpel and scissors dissection and sliced (0.5 mm thick) by using a Stadie-Riggs microtome. Papillary slices were collected in ice-cold 10 mM Tris–HCl, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$ and 5.5 mM glucose (TBS) and incubated at 37 °C in a metabolic shaking bath either in the absence or in the presence of membrane-affecting agents: 5 mM methyl-$\beta$-cyclodextrin or 1 mM neomycin or 10 mM LiCl for 30, 10 or 120 min, respectively. Incubations were stopped on ice and 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, 10 μg/mL aprotinin and 1 mM $Na_3VO_4$ and, successively centrifuged at 860 g for 10 min, 8,000 g for 20 min, and 105,000 g for 60 min; the resultant pellet corresponding to microsomal fraction was used for further studies.

### Detergent-Resistant Membrane Isolation

Triton X-100 insoluble membrane fractions were performed by the two-step centrifugation process described by Ito el al. [11]. Briefly, microsomes were resuspended in one vol of ice-cold PBS containing 1 mM PMSF, 10 μg/mL aprotinin and 1 mM $Na_3VO_4$. Then, one volume of 0.2% (v/v) Triton X-100 in PBS was added, mixed and incubated at 4 °C for 20 min. Samples were layered on 30% (w/v) sucrose and centrifuged at 225,000*g* at 4 °C for 2.5 h. Pellets were discarded and supernatants diluted with 0.1% Triton X-100 in PBS up to 10% (w/v) sucrose and centrifuged at 225,000*g* at 4 °C for 2.5 h. The resultant pellet contained the Triton X-100 insoluble membrane fraction while supernatants were considered as the soluble membrane fraction (S fraction). Throughout this paper we termed DRM as the 0.1% Triton X-100 insoluble membranes that sediment in 10% sucrose after centrifuging for 2.5 h at 225,000 g. For the biochemical studies we used DRM isolated from microsomal fractions instead of the plasmatic membrane because in our experimental conditions no detergent resistant pellet was obtained from the isolated endoplasmatic reticulum. Thus, our microsomal DRM only represented resistant domains from the plasmatic membrane.

### Lipid Analysis

Total lipids were obtained in the lower chloroformic phase of the Bligh and Dyer extraction procedure [12]. From total

lipid extracts, individual phospholipids and phosphoinositides were separated and quantified as previously reported [13, 14], and the cholesterol content determined by the method based on the cholesterol-oxidase enzyme reaction [15]. Phospholipid biosynthesis was studied by incubating papillary slices in TBS containing 0.1 μCi/mL of [$^{32}$P]-sodium orthophosphate ([$^{32}$P]-Pi) at 37 °C in a metabolic shaking bath. After 60 min incubation, samples were homogenized, DRM and S fractions obtained, and phospholipids and polyphosphoinositides separated. Radioactive lipids were visualized by autoradiography and iodine vapors and quantified by liquid scintillation counting.

## Fatty Acid Determination

Following phospholipid separation by TLC, spots were scraped off and stored under nitrogen atmosphere at −20 °C. The lipids were *trans*-esterified with acetyl chloride in methanol at 100 °C for 1 h. After cooling, $K_2CO_3$ was added, and the methyl esters were extracted with benzene. Thereafter, the extracts were evaporated under stream of nitrogen and stored at −20 °C. Methyl esters were analyzed by gas chromatography using a Gas Chromatograph (Series GC-8A) equipped with a flame ionization detector and 30 m × 0.25 mm DR-23 capillary column. The temperature was programmed from 175 to 240 °C at a rate of 3 °C/min. Nitrogen was used as the carrier gas. The chromatographic peaks were identified by comparison of the retention time with those of standards and the percentage composition of each fatty acid was calculated from the proportionality of peak areas by an automatic integrator.

## Phospholipid Determination

For the quantification of phospholipids, specific areas of the TLC plates were scraped off and digested with 70% perchloric acid in the presence of ammonium molybdate (0.5%), for 2 h in a heating block at 180 °C. The resulting inorganic phosphate was assayed with a Fiske–Subbarow reagent [16].

## Immunoprecipitation and Western Blot Analysis

Aliquots containing 100 μg of protein of DRM or S fractions were pre-cleared by incubation at 4 °C with protein A/G Plus-Agarose and, after centrifugation, supernatants were incubated at 4 °C for 1 h with 2 μg of monoclonal antibody against PtdIn(4,5)P2 (plus 2.5 μg of rabbit anti-mouse IgM) or monoclonal antibody against vinculin. Thereafter, protein A/G Plus- Agarose was added and incubated overnight at 4 °C with gentle shaking. The immunoprecipitates were washed three times with HNTG buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1 % (v/v) Triton X-100 and 10 % w/v glycerol), resuspended in Laemmli buffer and boiled for 5 min prior to Western blot analysis. Proteins were resolved in 8 % SDS-PAGE, electrotransferred to PVDF membranes and detected with the antibody of interest. Primary interaction was evidenced by using ECL Kit (GE Healthcare Life Sciences). When necessary, membranes were stripped and reprobed with the antibody of interest and evidenced with the ABC/HRP complex and 3, 3′diaminobenzidine. The intensity of each band was estimated by optical densitometry with a Gel-Pro Analyzer version 3.1 (Media Cybernetics, USA).

## Cell Culture and Fluorescence Microscopy

Primary culture of papillary collecting duct cells was performed by means of Stokes et al.'s procedure [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) with F-12 (1:1), 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 TBS containing 1 mM neomycin, 5 mM CD or 10 mM LiCl for 10, 30 min, or 24 h, respectively, and then the cover slips were transferred to cold PBS. For immunofluorescence labeling cells were washed with PBS and fixed with methanol (at −20 °C for 10 min) and acetone (at −20 °C for 4 min), blocked with 3% (v/v) goat normal serum in PBS. After fixation, cells were stained with mouse anti vinculin or mouse anti talin overnight at 4 °C in goat serum containing PBS. Detection of primary antibodies was performed with FITC conjugated F (ab́)$_2$ fragment goat anti-mouse IgG (Jackson Immuno Research) secondary antibody. Finally, cells were washed in PBS and mounted using Vectashield Mounting Media (Vector Lab) and stored at 4 °C until analysis. Specimens were examined with an Olympus FV300 Confocal Microscopy (Model BX61), with an acquisition software FluoView version 3.3 provided by the manufacturer. The confocal images were analyzed using the Image-Pro Plus version 5.1.2 software (Media Cybernetics, USA). All

adhesion sites (focal contacts) in 10–15 randomly selected cells were analyzed for generation of quantitative data sets for each treatment, and three independent experiments were performed. Cells were examined with a 100X Plan Apo oil objective (NA 1.4), resulting in a pixel length of 0.1388 μm after performing the spatial calibration. To even out background variations in our immunofluorescence images, we applied a "flatten filter". To select the intensity range of the objects (focal contacts) to be counted, we applied the software command to perform the "segmentation" of the image. The total number of vinculin- and talin- immunostained FC per cell was counted. To do so, the contour of each cell in the confocal image (area of interest) was manually drawn by using the mouse pointer of the program.

### Statistics

Results were expressed as mean ± SE. We used the unpaired *t*-test for comparison between DRM and S fractions. Data from control and different treatments were analyzed by ANOVA and significant differences were assessed by "a posteriori" Dunnett Multiple Comparisons test ($P < 0.05$).

## Results

### Renal Papillary Focal Contact (FC) Complexes are Localized in Specific Detergent-Resistant Membranes

We first evaluated the presence of FC proteins vinculin and talin in 0.1% Triton X-100 resistant (DRM) and soluble (S) fractions. As seen in Fig. 1a, anti-vinculin recognized a 116-kDa band in both fractions. By contrast, anti-talin recognized a 225-kDa band only in the DRM fraction (Fig. 1b). It is considered that PtdIns(4,5)$P_2$ is an essential lipid for FC stabilization [1, 2]; therefore, we next determined whether vinculin and talin were associated with PtdIns(4,5)$P_2$ in both DRM and S fractions. Thus, PVDF membranes were stripped and retested with anti PtdIns(4,5)$P_2$ antibody. Two bands were clearly detected in DRM but not in the S fraction (Fig. 1 C). The highest molecular mass band (∼225 kDa) was compatible with PtdIns(4,5)$P_2$ association with talin, while the second band (molecular mass ∼116 kDa) was consistent with PtdIns(4,5)$P_2$ bound to vinculin. In order to confirm whether vinculin and talin were, in fact, bound to PtdIns(4,5)$P_2$, we immunoprecipitated DRM and S fractions with anti-PtdIns(4,5)$P_2$ and then, vinculin and talin were detected by Western blot. In DRM, both vinculin and talin were pulled down by PtdIns(4,5)$P_2$ antibody, while no bands were evident in immunoprecipitates from the S fraction (Fig. 1d, e). To verify the presence of assembled FC complexes in DRM, talin–vinculin interaction was addressed by performing an immunoprecipitation with anti-vinculin and then, talin was detected by Western blot in the precipitate. As seen in Fig. 1f, a positive band (molecular mass ∼225 kDa) was obtained with anti talin, thus reflecting vinculin–talin association. The presence of vinculin bound to PtdIns(4,5)$P_2$, together with talin–vinculin association in DRM, constitute the biochemical expression of the existence of FC complexes in DRM isolated from rat renal papillae.

### Biochemical Characterization of FC-Containing DRMs

Once established that FC complexes were located in DRM, we characterized the DRM lipid composition in

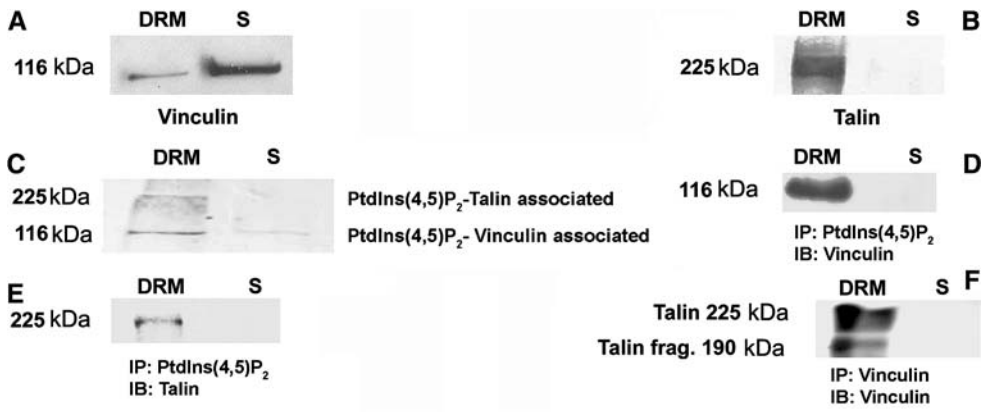


**Fig. 1** Biochemical protein-characterization of DRMs from renal papillary cells. DRM and S fractions were obtained from renal papillae as described in "Materials and Methods", and equivalent amounts of protein were resolved by electrophoresis and immunoblotted for vinculin (**a**) and talin (**b**). Representative membranes were stripped and evaluated for PtdIns(4,5)$P_2$ (**c**). DRM and S were immunoprecipitated (IP) for PtdIns(4,5)$P_2$ (**d**, **e**), and for vinculin (**f**) and then immunoblotted (IB) with the indicated antibody. Results correspond to a representative experiment of five individual assays

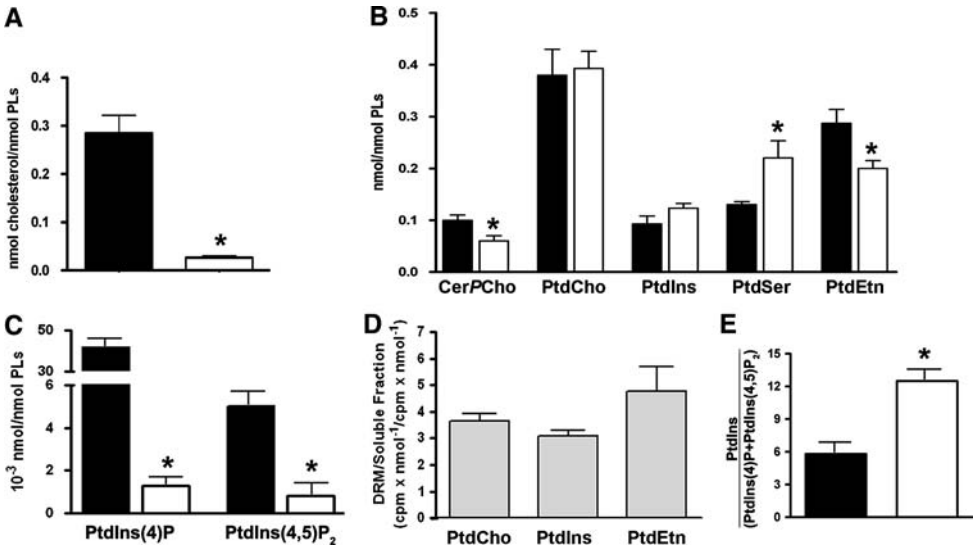


**Fig. 2** Lipid characterization of DRM (*black*) and S fractions (*white*). Endogenous content of cholesterol (**a**), individual phospholipids (**b**), and polyphosphoinositides (**c**) was determined on DRM and S aliquots, according to the methodology described for each kind of molecule. **d** [$^{32}$P]-phospholipids ratio and **e** [$^{32}$P]-PtdIns and [$^{32}$P]-PtdIns(4)$P_2$ plus [$^{32}$P]-PtdIns(4,5)$P_2$ ratio. (Mean ± SE, $n = 5$). *Significantly different from the S fraction, $P < 0.05$. *PLs* phospholipids

comparison with the S fractions. As seen in Fig. 2a, cholesterol concentration was ten times higher in DRM than in S fractions (Fig. 2a). With respect to the phospholipid profile (Fig. 2b), DRM showed a 70% increase in sphingomyelin (CerPCho), accompanied by a 30% increase in phosphatidylethanolamine (PtdEtn) and a 40% decrease in phosphatidylserine (PtdSer) but no differences were found in phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) concentrations. The study of individual phospholipid fatty acid composition revealed that DRM are enriched in arachidonic acid mainly due to PtdEtn, which doubled its content with respect to PtdEtn in S fractions (Table 1). The amount of DRM-polyphosphoinositides, PtdIns(4)P and PtdIns(4,5)$P_2$, was several times higher than in the S fraction, denoting the massive accumulation of these acidic phospholipids in our papillary DRM (Fig. 2c). In order to determine whether or not DRM-associated phospholipids were metabolically active, we evaluated [$^{32}$P]-Pi incorporation into phospholipids. [$^{32}$P]-Pi specific activity, expressed as [$^{32}$P]-Pi per nmol of each phospholipid, was obtained and the DRM/S ratio calculated for each individual phospholipid. As seen in Fig. 2d, [$^{32}$P]-Pi incorporation was more than three times higher in DRM than in S fraction. It is known that PtdIns(4)P and PtdIns(4,5)$P_2$ are formed by PtdIns phosphorylation by specific kinases. As a reflection of such a dynamic process, we analyzed the ratio between [$^{32}$P]-PtdIns and [$^{32}$P]-PtdIns(4)P plus [$^{32}$P]-PtdIns(4,5)$P_2$ in DRM and S

**Table 1** Fatty acid profile of DRM and soluble fraction phospholipids

| | CerPCho | | PtdCho | | PtdIns | | PtdSer | | PtdEtn | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DRM (%) | S (%) | DRM (%) | S (%) | DRM (%) | S (%) | DRM (%) | S (%) | DRM (%) | S (%) |
| 14:0 | 4.6 | 4.0 | 1.2 | 2.0 | 4.5 | 5.0 | 4.2 | 4.5 | 1.7 | 2.8 |
| 16:0 | 37.7 | 37.4 | 27.5 | 33.0 | 32.9 | 35.3 | 28.8 | 33.4 | 17.7 | 21.0 |
| 16:1 | 1.6 | 1.7 | | 1.3 | 1.3 | 1.9 | 1.4 | 1.3 | | 2.4 |
| 17:0 | 1.7 | 1.8 | 1.5 | 1.4 | 1.7 | 1.6 | 1.5 | 1.5 | 1.1 | 2.7 |
| 18:0 | 33.8 | 34.8 | 21.8 | 26.0 | 40.8 | 38.8 | 40.3 | 40.5 | 30.5 | 25.8 |
| 18:1 | | 1.4 | 16.6 | 12.5 | | 2.1 | 6.1 | 3.4 | 5.6 | 6.4 |
| 18:2 | | | 10.9 | 8.1 | | | 1.9 | 1.1 | 3.6 | 1.9 |
| 18:3 | | | | | | | | | | 2.6 |
| 20:0 | 1.7 | 1.8 | 0.9 | 0.9 | | | 1.0 | | | 2.9 |
| 20:2 | | 0.8 | 0.4 | | | | | | | |
| 20:4 | | | 14.7 | 11.3 | | 0.7 | 6.2 | 4.1 | 33.1 | 16.0 |
| 22:0 | 4.5 | 4.1 | 0.7 | 0.9 | 7.9 | 4.9 | 1.6 | 2.3 | 1.0 | 2.2 |
| 22:1 | | 0.5 | | | 2.1 | 2.3 | | | | |
| 23:0 | 1.0 | 0.8 | | | | | | | | |
| 24:0 | 4.6 | 3.4 | | | | | | | 2.1 | |
| 26:0 | | | | | | | | | | 3.5 |

Phospholipids present in both DRM and soluble fraction were isolated and specific fatty acid profile was determined for each phospholipid as described in "Materials and Methods". The table shows the results of one representative experiment ($n = 3$)

fractions. As seen in Fig. 2e, the DRM ratio was around half of the S fraction ratio, reflecting that polyphosphoinositide synthesis in DRM is twice higher than in the S fraction.

Altogether, these results demonstrate that FC complexes are located in cholesterol-CerPCho-polyphosphoinositide-enriched membrane domains, which show an exceptionally high phospholipid biosynthesis with respect to the S fraction (Fig. 2d). The high polyunsaturated PtdEtn content, plus the enrichment in polyphosphoinositides, suggests that such DRM correspond to the inner leaflet of the plasma membrane, which is consistent with the fact that FC are the cytoplasmic face of the cell contact site [18].

## Effect of Membrane-Affecting Agents in DRM Compositions

Methyl-$\beta$-cyclodextrin (CD), a known cholesterol-depleting agent, decreased cholesterol content by 35%, and also induced an overall change in phospholipid profile (Fig. 3a). The most significant change was the great augmentation in CerPCho and PtdIns, counterbalanced by the decrease in PtdCho and PtdEtn content, while PtdIns(4)P and PtdIns(4,5)$P_2$ did not significantly change.

Neomycin, a known PtdIns(4,5)$P_2$ sequestering agent [19, 20], provoked an unexpected 60% loss in cholesterol concentration, which was accompanied by a slight decrease in CerPCho and PtdSer, and an increase in PtdEtn content. Neomycin also increased PtdIns(4,5)$P_2$ by 65% while PtdIns(4)P concentration fell about 10% (Fig. 3b).

The treatment with LiCl, which blocks phosphoinositides synthesis [21], evoked an increase in cholesterol and CerPCho content by 35 and 25%, respectively, accompanied by a decrease in PtdCho, an important PtdIns and PtdSer enrichment. No changes in PtdEtn content were observed. Both PtdIns(4)P and PtdIns(4,5)$P_2$ contents were diminished, accounting for a 50 and 25% decrease, respectively (Fig. 3c).

These results show that instead of evoking an effect on a single membrane lipid, each membrane-affecting agent appears to induce an unexpected overall change in the DRM-lipid profile, thus bringing about a DRM of different lipid composition.

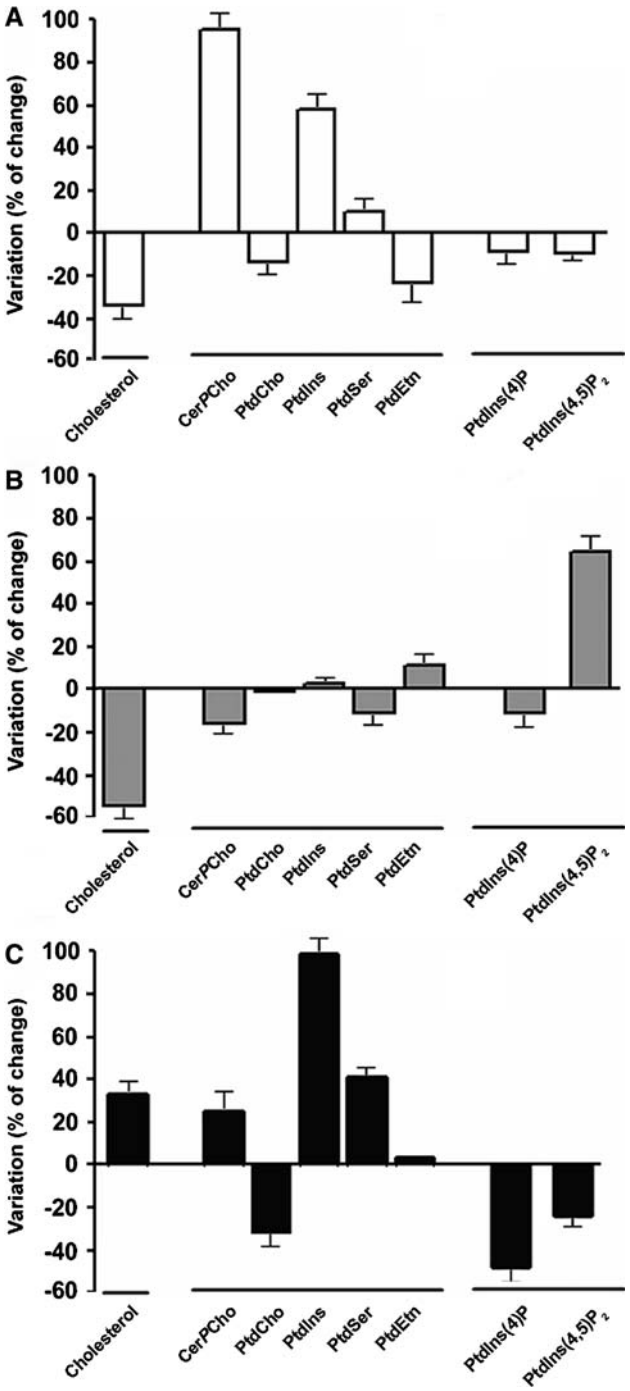


**Fig. 3** Changes in lipid composition of DRMs after treatment with membrane-affecting agents. Renal papillary slices were treated with **a** 5 mM CD, **b** 1 mM Neomycin, or **c** 10 mM LiCl, and DRMs were isolated. Variation of cholesterol, individual phospholipids, and polyphosphoinositides is expressed as percentage of control (Mean ± SE, $n = 5$)

## Influence of Changes in DRM Lipid Composition on FC Maintenance

In order to obtain a morphological correlation of the biochemical findings, we performed primary cultures of collecting duct cells and studied the FC by immunofluorescence microscopy. As shown in Fig. 4a, vinculin-stained FC appeared as bright and elongated structures (arrowheads). Vinculin immunoreactivity was also observed diffusively distributed in the cytoplasm. By contrast, talin immmunoreactivity was exclusively found in

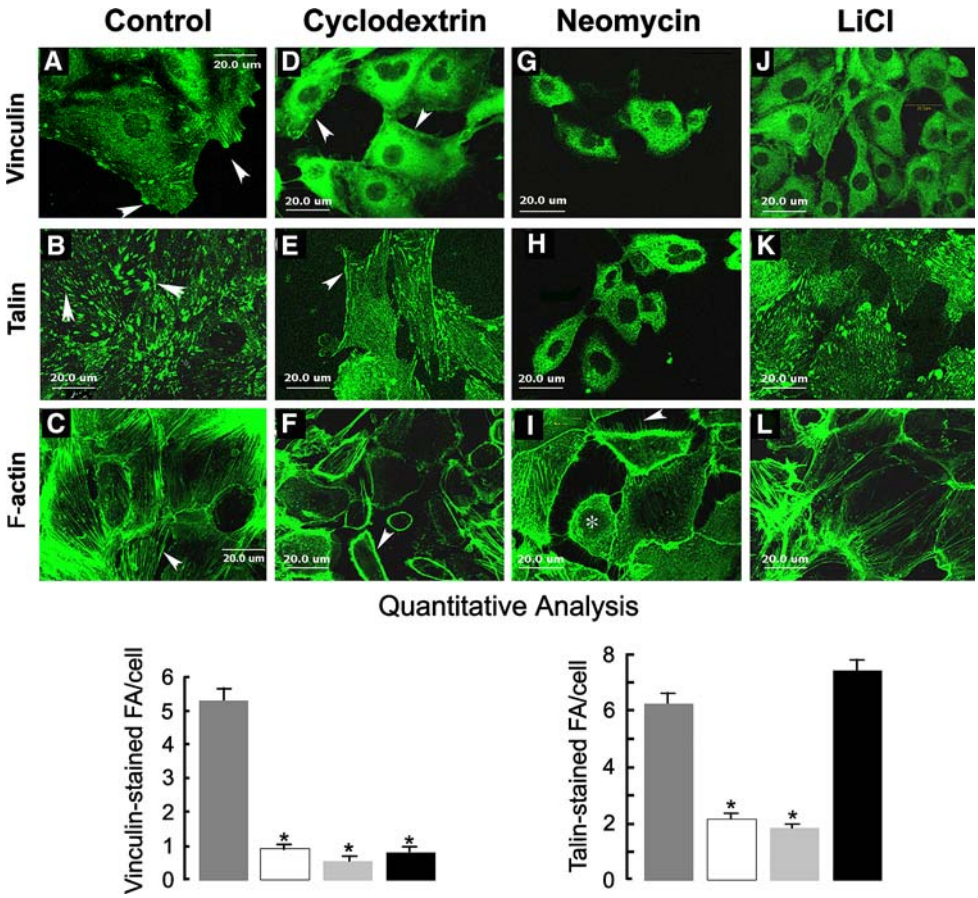


**Fig. 4** Effect of changes in DRM lipid composition on FC plaques. Cultured collecting duct cells were treated with 5 mM CD, 1 mM Neo, or 10 mM LiCl. After fixation, cells were immunostained with antibodies against vinculin (**a**, **d**, **g**, **j**) and talin (**b**, **e**, **b**, **k**), and with FITC-phalloidin (**c**, **f**, **i**, **l**). Primary monoclonal interactions were evidenced by using goat anti-mouse IgG–FITC secondary antibody and analyzed with confocal microscopy. Representative images of three independent experiments are shown. *Scale bar* 20 μm. Quantization of the number of vinculin-stained, and talin-stained FC per cell were calculated by the image analysis program, and performed as described in "Materials and Methods". *Bar graphs* results are expressed as mean ± SE. *Significantly different from control, $P < 0.01$

FC, appearing as abundant bright plaques morphologically heterogeneous (Fig. 4b, arrowheads).

Treatment of cultured cells with CD caused dissipation of vinculin-stained FC (Fig. 4 and quantitative analysis). Immunoreactivity increased in discrete zones of the plasma membrane (arrowhead) and was also accumulated in the perinuclear zone (Fig. 4d). Talin-stained FC were also affected by CD and an unusual accumulation in specific zones of the plasma membrane was also observed (Fig. 4e, arrowhead and quantitative analysis). Taken together, these results demonstrate that although a fraction of vinculin and talin remained in the plasma membrane, the assembly of these proteins in FC was almost completely impaired by CD. Consistent with the dissipation of FC, CD induced almost complete disappearance of stress fibers. F-actin appeared as a cortical network in rounded cells (Fig. 4f, arrowhead), typical of isolated–detached cells but not of cells that constitute organized epithelial tissues.

As seen in Fig. 4g and quantitative analysis, the specific PtdIns(4,5)$P_2$ sequestering agent neomycin induced almost complete loss of both vinculin- and talin-stained FC. Both proteins appeared densely accumulated in the cytosol with a punctuate appearance neighboring the nucleus (Fig. 4g, h). Consistent with the dissipation of FC, stress fibers almost disappeared and arrangements of peripherical actin-based structures were evident (Fig. 4i, arrowhead). Phalloidin-stained images also allowed us to visualize cells that were in the process of retraction (Fig. 4i, asterisk).

The treatment of culture cells with LiCl induced a decrease in the number of vinculin-stained FC per cell (Fig. 4j and quantitative analysis). By contrast, neither the quantity of talin-stained FC nor F-actin distribution in cultured cells was affected (Fig. 4k, l, and quantitative analysis). The preservation of actin organization in stress fibers explains the maintenance of cellular morphology observed after LiCl treatment (Fig. 4l).

## Discussion

In the present report, we biochemically studied FC complexes obtained from rat renal papillae and then defined their morphological correlation in papillary collecting duct cells primary cultures. By using a two-step centrifugation procedure and changing sucrose concentration, we were able to demonstrate that FC complexes are part of a detergent resistant membrane fraction, highly enriched in cholesterol and phosphoinositides and whose phospholipid constituents are subjected to a very high turn-over.

FC has been previously associated with detergent resistant domains and also with local membrane accumulation of PtdIns(4,5)$P_2$ [22] and it has been recently shown that they can regulate membrane order [23], thus supporting the notion that actin-containing cytoskeleton can promote lateral segregation of cholesterol–sphingomyelin enriched detergent resistant domains [24]. However, a direct demonstration of the existence of such resistant domains and their biochemical characterization has not been reported before. Here, we were able to detect the FC complex in a Triton X-100 resistant lipid domain and we propose that the lipid composition of such DRM corresponds to the physiological environment where FC are inserted.

For the isolation of DRM we used a low Triton X-100 concentration (0.1%). It has been demonstrated in stomach smooth muscle that at such a detergent and similar sucrose concentration most of vinculin was efficiently solubilized while sphingomyelin remained insoluble [24]. Consistent with this observation, we also found the bulk of vinculin in the soluble fraction, but this fraction of vinculin did not correspond to the FC-vinculin since it did not interact with PtdIns(4,5)$P_2$ nor with talin. Moreover, talin, which is immediately degraded by calpain if it is not assembled into FC, was only found in the detergent resistant domain. Since vinculin, besides being part of FC, can also form adherent junctions, it is possible that the bulk of the solubilized vinculin corresponds to vinculin present in adherent junctions, as suggested previously [23].

FC cannot be detected in histological preparations from intact tissue, but they can be observed in cultured cells. Therefore, we took advantage of the fact that cultured papillary collecting duct cells preserve their tendency to interact with their self-formed ECM, mimicking their behavior in intact tissue. Therefore, we established a parallelism between the biochemical data obtained from papillary microsomes and the morphological observations from immunofluorescence microscopy performed in primary cultures of collecting duct cells. By using such experimental strategy, we demonstrated that membrane lipid composition affects the in vivo preservation of FC as clearly shown by the immunofluorescence images. At first, we showed that the various membrane-affecting agents differently affect FC, depending on their capacity to change the membrane lipid composition. Consistent with previous observations in smooth muscle cells [24], the treatment with CD did not lead to DRM elimination. Instead, CD induced cholesterol decrease but also membrane lipid redistribution, which resulted in a net increase of the CerPCho content in DRM. It has been reported that DRM serves as a platform for PtdIns(4,5)$P_2$ accumulation induced by FC proteins [22], and that such PtdIns(4,5)$P_2$ accumulation serves to stabilize the assembled FC [6]. However, here we show that, although polyphosphoinositides were still accumulated in CD-treated DRMs, FC did not persist assembled as seen in the immunofluorescence images. Based on these observations, we can suggest that polyphosphoinositide accumulation in DRM is not sufficient to stabilize FC; but polyphosphoinositides must be included in a DRM with a specific lipid composition in order to guarantee an efficient FC stabilization.

The effect of neomycin on cytoskeleton organization has currently been attributed to a failure in the PtdIns(4,5)$P_2$ availability to bind cytoskeletal proteins but the membrane lipid composition was not studied [22]. The present results clearly show that apart from its well known action as PtdIns(4,5)$P_2$ sequestering-agent, neomycin simultaneously evokes a dramatic decrease in the DRM cholesterol content. Therefore, we suggest that the deleterious effect of neomycin on FC can be due not only to the loss of PtdIns(4,5)$P_2$ availability but also to a decrease in cholesterol content, which with no doubt changes the physico-chemical properties of the new formed DRM.

It has been previously shown that LiCl dissipates FC in neural cells [22]. However, here we show that the inhibition of polyphosphoinositide synthesis by LiCl induces a restructuration rather than a dissipation of FC. It is known that FC are hierarchical structures where additional FC proteins assemble as they mature. Talin is the first protein that forms FC, while vinculin is added thereafter [25]. Thus, even without vinculin, talin-containing FC can still be present, thus conforming the minimal FC assembling. Thus, the apparent controversy with the previous observations [22] resides in the fact that the authors studied only vinculin-stained but not talin-stained FC. It is known that vinculin has to be bound to PtdIns(4,5)$P_2$ to stay in FC plaques [26]. The selective dissipation of vinculin from assembled FC can be explained considering their different affinities for membrane lipids. Thus, vinculin binding to PtdIns(4,5)$P_2$ is of low affinity, while binding to PtdIns is of high affinity but does not serve to assemble vinculin into FC [27]. Thus, the lowering in PtdIns(4,5)$P_2$ content, together with the high increase in PtdIns produced by LiCl, may account for the dissipation of vinculin from FC. By contrast, talin has high affinity to bind PtdIns(4)P [27], thus the decreased concentration of DRM–PtdIns(4)P, could be still enough to bind talin. On the other hand, it appears that the new DRM conformation, where cholesterol and CerPCho increased their concentration, could result favorable to stabilize talin-containing FC. Moreover, it has been reported that LiCl affects 20:4n-6 turnover by altering the expression of $cPLA_2$ [28]; however, whether or not such effect is also involved in the dissipation of vinculin-containing FC needs further demonstration.

Investigations on the interaction of the cytoskeleton components with artificial membranes, and in more detail with membrane lipids were started in the 1980s [29]. However, no direct evidence on the effect of changes in the membrane lipid composition in biological membranes on FC structures exists. It is currently considered that PtdIns(4,5)$P_2$ is the membrane phospholipid that plays the crucial role in the maintenance of assembled FC. However, our results demonstrated that such pool of polyphosphoinositides has to be part of a domain of specific lipid composition to serve as a membrane lipid stabilizing FC plaques. Among membrane lipids, and taking in consideration that the decrease in cholesterol was a common feature in the deleterious effect of CD and Neo, we suggest that the enrichment in cholesterol is in fact the crucial lipid for evoking the lipid environment to maintain the FC plaques assembled.

We used the various membrane affecting agents only as a tool to study the influence of membrane lipid composition on FC maintenance. However, it is of interest to point out that both neomycin and LiCl are pharmacological agents of known nephrotoxic effects. In this context, our results could be also pharmacologically relevant. Indeed, neomycin is an aminoglycoside antibiotic known to cause tubular necrosis [30, 31]. Thus, the disruption of the cell-extracellular matrix adhesion demonstrated by the present data could be an explanation for the deleterious effect caused by neomycin treatments since it is known that epithelial cells have to be bound to the extracellular matrix in order to survive and not die by anoikis. On the other hand, LiCl is used for the treatment of some human mental diseases [32, 33] and it is known that long-term treatment with this agent provokes alterations in the renal capacity for concentrating urine [34]. Although the pharmacological dose is lower (0.8–1 mM) than the concentration that we used, chronic low doses could also affect FC. The impairment of vinculin-contained FC reported here could be a primary cause that progressively affects cell-ECM adhesion, thus affecting renal function. Moreover, the alteration in membrane lipid composition, mostly cholesterol enrichment, could disturb the location or the function of membrane transporter systems involved in normal renal tubular physiology, thus affecting the urine concentration process.

In conclusion, in this work we show evidence about the importance of the lipid composition of the specific membrane lipid domain, where FC are included, and suggest that it could be relevant for the maintenance of the structures that tether the collecting duct epithelial cells to the extracellular matrix.

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