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Changes in membrane lipid composition cause alterations in epithelial cell–cell adhesion structures in renal papillary collecting duct cells

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ABSTRACT

In epithelial tissues, adherens junctions (AJ) mediate cell–cell adhesion by using proteins called E-cadherins, which span the plasma membrane, contact E-cadherin on other cells and connect with the actin cytoskeleton inside the cell. Although AJ protein complexes are inserted in detergent-resistant membrane microdomains, the influence of membrane lipid composition in the preservation of AJ structures has not been extensively addressed. In the present work, we studied the contribution of membrane lipids to the preservation of renal epithelial cell–cell adhesion structures. We biochemically characterized the lipid composition of membranes containing AJ complexes. By using lipid membrane-affecting agents, we found that such agents induced the formation of new AJ protein-containing domains of different lipid composition. By using both biochemical approaches and fluorescence microscopy we demonstrated that the membrane phospholipid composition plays an essential role in the in vivo maintenance of AJ structures involved in cell–cell adhesion structures in renal papillary collecting duct cells.

1. Introduction

The adhesion of cells to one another as well as to the extracellular matrix is essential to the maintenance of tissue integrity. Adherens junctions (AJs) mediate cell–cell adhesion using proteins called cadherins. Cadherins span the cell membrane, contacting cadherins on other cells in a Ca^{+2}-dependent homophilic manner and linking to the actin cytoskeleton inside the cell [1]. In epithelial tissues, E-cadherin is organized in a "core complex" that includes E-cadherin itself, α-, β-, and γ-catenins, α-actinin, and vinculin. β-catenin interacts directly with the cytoplasmic tail of E-cadherin and connects E-cadherin to α-catenin, which binds F-actin. α-actinin and vinculin are also F-actin-binding proteins that bind directly to α-catenin [1]. The integrity of this core complex is critical to the formation and/or maintenance of stable cell–cell adhesions [2,3].

Many different cellular processes, such as migration, proliferation and differentiation, can affect cell adhesion. These processes affect AJs at different levels and, therefore, could be regulated by different mechanisms. The small GTPases Rac, Rho, and Cdc42 have been involved in cadherin-mediated adhesions [1,4]. Tyrosine phosphorylation of the cadherin–catenin complex has also been involved in the regulation of AJ assembly [5,6]. In contrast to the extensive documentation on the mechanisms underlying the formation and maturation of AJs, the role of the membrane lipidic environment in the regulation of such cell–cell adhesion structures has been less often studied.

Cytoskeleton–lipid interactions seem to be involved in mediating the anchorage of the cytoskeleton in the membrane bilayer as well as in defining the architecture of specific membrane areas, such as membrane rafts [7,8]. The concept of membrane raft microdomains was first used more than ten years ago [9–11]. These membrane microdomains are rich in cholesterol and sphingolipids, and are resistant to solubilization by non-ionic detergents such as Triton X-100 in cold [10,12]. Thus, after extraction of cells in detergent, insoluble rafts can be separated from solubilized non-raft proteins and lipids for further analysis. While raft refers to the domain in the intact membrane, the term DRM (detergent-resistant membranes) corresponds to the structure isolated by detergent insolubility [12]. Rafts localize in the plasma membrane, and can also be found in other membrane compartments such as the Golgi apparatus and in the endocytic pathway [10,13]. It has been reported that many of the molecular components that regulate actin cytoskeleton and cell adhesion structures are associated with rafts [7,8,14,15]. Thereafter, the study of the consequences of changes in the lipidic composition of the membrane environment—where cell adhesion and cytoskeleton-associated proteins are anchored—is of relevance in a cellular context.

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Recently, we have demonstrated that membrane lipid composition plays a central role in the maintenance of renal papillary collecting duct cell adhesion to the extracellular matrix [16]. In the present work, we studied the role of membrane lipid composition in the maintenance of renal papillary collecting duct cell adhesion to each other. First, we biochemically characterized the membrane domains where AJ protein complexes are localized in renal papillary cells and then studied the effect of changes in the lipidic composition of such microdomains on AJ structure and composition. By combining biochemical and immunofluorescence studies, we present experimental evidence suggesting that in collecting duct cells, the specific lipidic composition of rafts is a requisite for AJ maintenance, necessary to assure a correct attachment of collecting duct cells to each other.

2. Materials and methods

2.1. Animals and tissue preparation

Male Wistar rats (250–300 g) were housed in a light-controlled room with a 12:12 h light–dark cycle and allowed free access to water and standard rat chow. All animals were handled according to the rules for animal care and use of laboratory animals of the University of Buenos Aires (*Reglamento para el cuidado y uso de animales de laboratorio en la Universidad de Buenos Aires*). The animal protocol was reviewed and approved by the Comité de Ética para el Cuidado y Uso de Animales de Laboratorio de la Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (CICUAL-FFYB). Rats were killed by decapitation, kidneys were removed, and renal papillae 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 (Tris-buffered saline, 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-β-cyclodextrin (Sigma, Saint Louis, MO, USA), 1 mM neomycin (Sigma, Saint Louis, MO, USA), 25 μM lovastatin (Calbiochem-Merck, Darmstadt, Germay), or 10 mM LiCl (Sigma, Saint Louis, MO, USA), for 30, 10, 120, or 120 min, respectively. Incubations were stopped on ice and immediately homogenized in 10 vol of a solution of 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. Aliquots from the resulting homogenates were used to study the total level of AJ proteins. The rest of the homogenates were successively centrifuged at 860 *g* for 10 min, 8000 *g* for 20 min, and 105,000 *g* for 60 min; the resultant pellet corresponding to the microsomal fraction was used for further studies.

2.2. Detergent-resistant membrane isolation

Triton X-100 insoluble membrane fractions were obtained by the two-step centrifugation process as previously described [17]. 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 to remove the membranes protein–lipid complex as a pellet [17]. The supernatants were diluted by three folds with PBS containing same protease inhibitors to make the sucrose concentration 10% (w/v) and centrifuged at 225,000 *g* at 4 °C for 2.5 h. The resulting pellet contained the Triton X-100 insoluble membrane fraction and 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 sedimented in 10% sucrose after centrifuging for 2.5 h at 225,000 *g*. Aliquots from homogenates, DRM and S fractions were assayed for protein content by the method of Lowry.

2.3. Lipid analysis

Total lipids from DRM and the S fraction were obtained in the lower chloroformic phase of the Bligh and Dyer extraction procedure [18]. From total lipid extracts, individual phospholipids were separated and quantified as previously reported [19], and cholesterol content determined by the method based on cholesterol-oxidase enzyme reaction [20]. For the quantification of phospholipids, specific areas of the TLC plates (Merck, Darmstadt, Germany) 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 [21].

2.4. Immunoprecipitation and Western blot analysis

For immnunoprecipitation purposes, aliquots of DRM containing 100 μg of protein of DRM were pre-cleared by incubation at 4 °C with protein A/G Plus-Agarose (Santa Cruz, California, USA), and, after centrifugation, supernatants were incubated at 4 °C for 1 h with 2 μg of monoclonal antibody against β-catenin (Sigma, Saint Louis, USA), or rabbit antibody against α-catenin (Sigma, Saint Louis, USA). 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. For Western blotting analysis of total homogenates, DRM and S fractions, sample aliquots containing 20 μg proteins were used. Proteins were resolved in 8% SDS-PAGE, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. After blotting, membranes were treated with 5% nonfat milk in TBS-Tween 20 and incubated with mouse anti-β-catenin (Sigma, Saint Louis, USA), rabbit anti-α-catenin (Sigma, Saint Louis, USA), and rabbit anti-E-cadherin (Santa Cruz, California, USA). Primary interaction was evidenced by using the enhanced chemiluminescence kit (GE Healthcare—Argentina Life Sciences, Buenos Aires, Argentina). When necessary, membranes were stripped and reprobed with the antibody of interest and evidenced with avidin–biotin-peroxidase (Dako, Glostrup, Denmark) and 3,3′diaminobenzidine (Sigma, Saint Louis, USA). To control the protein loading of samples, membranes were stained with Ponceau S. The intensity of each band was estimated by optical densitometry with a Gel-Pro Analyzer version 3.1 (Media Cybernetics, USA).

2.5. Cell cultures and treatments with lipid-affecting agents

Primary cultures of papillary collecting duct cells were performed according to Stokes et al. [22]. Briefly, renal papillae were minced to 1–2 mm^3 pieces and incubated at 37 °C in sterile TBS containing 0.1% collagenase II (Sigma, Saint Louis, USA) under 95%O_2/5%CO_2. After 40 min, digestion was stopped and isolated cells and structures were separated by centrifuging at 175 *g* for 10 min. The crude pellet containing most papillary cell types, tubular structures and tissue debris was washed twice and resuspended in Dulbecco's modified Eagle's medium (DMEM) with F-12 (1:1) (GIBCO, Invitrogen, California, USA), 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen, California, USA). The enriched collecting duct pellets were obtained by centrifugation at 60 *g* for 1 min and resuspended in an adequate volume of DMEM/F12. Enriched-tubular suspensions were seeded in sterile dry-glass coverslips placed in six-well multidishes. After growing at 37 °C for 96 h, cultures were treated with either 5 mM methyl-β-cyclodextrin for 30 min, 10 mM neomycin for 10 min, 25 μM lovastatin, or 10 mM LiCl for 24 h. Incubations were stopped on ice and then the coverslips were transferred to cold PBS.

2.6. Cell labeling and immunofluorescent microscopy

For immunostaining, cultured cells treated with methyl-β-cyclodextrin, neomycin, lovastatin or LiCl, as described above, were fixed with methanol (at −20 °C for 10 min) and acetone (at −20 °C for 4 min), and blocked with 3% goat normal serum (Vector Laboratories, California, USA) in PBS. Then, cells were incubated with the appropriate combinations of antibodies overnight at 4 °C in goat serum containing PBS. The following primary antibodies were used: mouse anti-β-catenin (Sigma, Saint Louis, USA), rabbit anti-α-catenin (Sigma, Saint Louis, USA), rabbit anti-E-cadherin (Santa Cruz, California, USA). Mouse and rabbit primary antibodies were detected using fluorescent Alexa Fluor 488 or 546 conjugated $F(ab)_2$ fragment goat anti-mouse or anti-rabbit (Molecular Probes). Finally, the cells were mounted using Vectashield Mounting Media (Vector Laboratories, California, USA) and stored at 4 °C until analysis. Specimens were examined with an Olympus FV300 Confocal Microscope (Model BX61), with an acquisition software FluoView version 3.3 provided by the manufacturer. Double fluorescence for green and red channels was visualized by using an argon–helium–neon laser. Double-stained images were obtained by sequential scanning for each channel to eliminate the crosstalk of chromophores. Analysis of the confocal images and the colocalization was performed with the image analysis software Image-Pro Plus version 5.1.2 (Media Cybernetics, USA). Cell morphology was assessed by F-actin staining with FITC-coupled phalloidin (Sigma, Saint Louis, USA) and by phase-contrast microscopy using an Olympus inverted microscope with an X40 objective. All images were obtained with a cooled CCD camera and processed for output purposes using Adobe Photoshop software.

2.7. Statistic

Results are expressed as mean ± SE. We used the unpaired *t*-test for comparison between DRM and S fractions, and between data from control and different treatments ($P<0.05$).

3. Results

3.1. Adherens junction proteins are localized in rafts microdomains in renal papillary collecting duct cells

To determine whether the AJ components E-cadherin, β-catenin, and α-catenin are part of DRM, we analyzed Triton X-100-resistant and soluble fractions obtained from the microsomal fraction of rat renal papillary collecting duct cells. DRM isolated in our experimental conditions probably represents a mix of various lipid rafts in the renal papillary collecting duct cells, of which only a part constitutes the AJ membrane domains. Immunoblot analysis of DRM and the soluble fraction showed bands corresponding to the molecular weight of E-cadherin (~125 KDa), α-catenin (~105 KDa) and β-catenin (~94 KDa). In the soluble fraction, no E-cadherin and α-catenin bands were detected, whereas a band corresponding to the molecular weight of β-catenin (~94 KDa) was observed (Fig. 1A).

In organized simple epithelia, like in collecting ducts, adherens junction E-cadherin is associated with catenins in the cytoplasmic domain, where they form a multiprotein complex in the sequence of cadherin to β-catenin, and β-catenin to α-catenin [1]. To verify whether such organized complex is present in the DRM microdomain, we performed an immunoprecipitation with anti-β-catenin and the presence of E-cadherin was analyzed in the precipitate. In separate samples, an immunoprecipitation with anti-α-catenin was performed and the presence of β-catenin was analyzed in the precipitate. As seen in Figs. 1B and C, E-cadherin was efficiently immunoprecipitated with anti-β-catenin, and β-catenin was immunoprecipitated with anti-α-catenin. The percentage of isolated E-cadherin immunoprecipitated by anti-β-catenin was of 57% while the percentage of β-catenin immunoprecipitated by anti-α-catenin was of 17% The fact that β-catenin was found bound to E-cadherin, and that α-catenin co-immunoprecipitated with β-catenin in Triton X-100 insoluble fraction denotes that AJ complexes are present in rafts microdomains in renal papillary collecting duct cells. It is interesting to note that the ratios between upper and lower bands in the immunoblot of E-cadherin before and after the immunoprecipitation with anti-β-catenin are different. It could be due to the presence of a calpanin induced E-cadherin fragment which is recognizable by the E-cadherin antibody but has lower affinity to bind β-catenin.

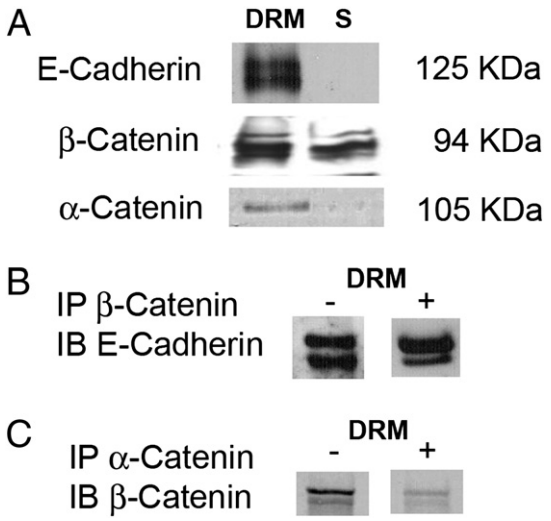

Fig. 1. Biochemical protein characterization of DRMs from renal papillary cells. DRM and Triton-soluble fractions (S) were obtained from renal papillae as described in Materials and methods, and equivalent amounts of protein were resolved by electrophoresis and immunoblotted for E-cadherin, β-catenin and α-catenin (A). DRM was immunoprecipitated (IP) for β-catenin (B), and for α-catenin (C) and then immunoblotted (IB) with the indicated antibody. Minus (−) indicates samples of DRM which were not immunuprecipitated with the indicated antibody. Results correspond to a representative experiment of three individual assays.

We further characterized the lipid composition of DRM in comparison with the Triton X-100 soluble (S) fraction, by determining the cholesterol and sphingomyelin (CerPCho) content, as well as the phospholipid profile. Typical of DRM, cholesterol and CerPCho concentration were higher in the insoluble than in the S fraction (Fig. 2A). With respect to the phospholipid profile, DRM showed a 30% increase in phosphatidylethanolamine (PtdEtn) and a 40% decrease in phosphatidylserine (PtdSer), with no differences in phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) concentrations (Fig. 2A). It is known that polyphosphoinositides play a central role in interacting with cytoskeleton proteins, thus regulating actin cytoskeleton dynamics [23]. The amounts of DRM-polyphosphoinositides, phosphatidylinositol-4-phosphate (PtdIns(4)P) and phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$), were several times higher than in the S fraction, denoting the massive accumulation of these acidic phospholipids in DRM (Fig. 2B). Altogether, these results demonstrate that in renal papillary collecting duct cells, AJ complexes are located in cholesterol–sphingomyelin–polyphosphoinositide-enriched membrane domains.

3.2. The persistence of AJ depends on membrane lipid composition

In order to study the importance of DRM lipid composition in the maintenance of the AJ complex, we treated renal papillary slices with the membrane-affecting agents methyl-β-cyclodextrin (CD), neomycin (Neo) and LiCl. We first checked changes in the lipid composition of the Triton insoluble fraction and thereafter we biochemically studied the presence of the AJ complex. We also evaluated whether the different treatments altered the total protein level in the DRM fraction isolated from the microsomal fraction.

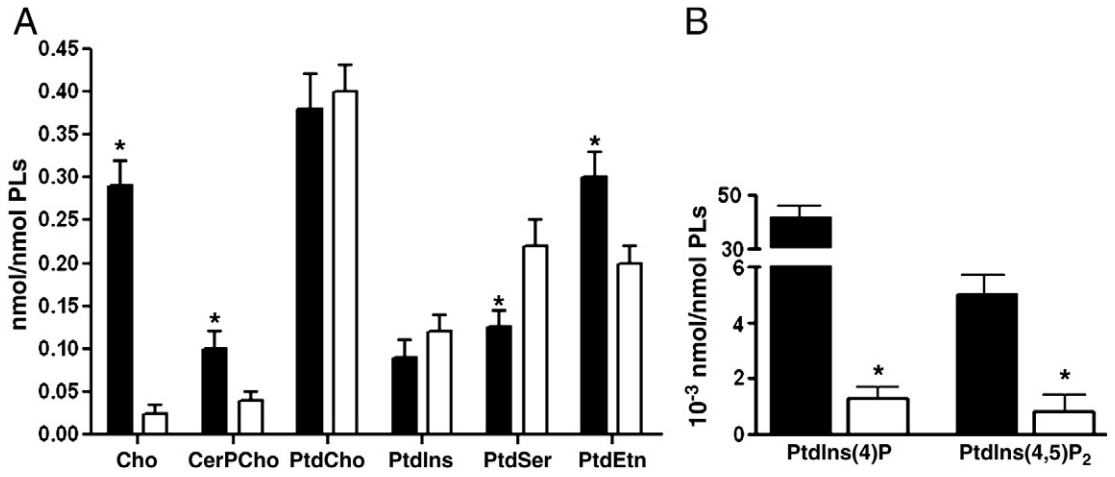

Fig. 2. Lipid characterization of DRM (black) and S fractions (white). Endogenous content of cholesterol, individual phospholipids (A), and polyphosphoinositides (B) was determined on DRM and S fraction aliquots, according to the methodology described for each kind of molecule. (Mean ± SE, n = 5). *Significantly different from S, $p<0.05$. PLs, phospholipids.

As shown in Fig. 3A, CD, a known cholesterol-depleting agent that selectively extracts cholesterol from the membrane without affecting the viability of living cells [24], decreased cholesterol content by 35%, but also caused an overall change in the phospholipid profile. The most significant change in the phospholipid profile was the 100% and 50% increase observed in CerPCho and PtdIns concentrations respectively, and the 30% decrease in PtdEtn content, while PtdIns(4)P and PtdIns(4,5)P_2 did not change significantly (Fig. 3A). Neo, a known PtdIns(4,5)P_2 sequestering agent [25], caused an unexpected 60% decrease in cholesterol concentration, accompanied by a slight decrease (10%) in CerPCho content (Fig. 3B). Neo also increased PtdIns(4,5)P_2 by 65% while PtdIns(4)P concentration decreased about 10% (Fig. 3B). The treatment with LiCl, which blocks phosphoinositide synthesis [26], evoked an increase in cholesterol and CerPCho content by 35% and 25%, respectively, accompanied by a decrease in PtdCho, and an important PtdIns and PtdSer enrichment. No changes in PtdEtn content were observed.

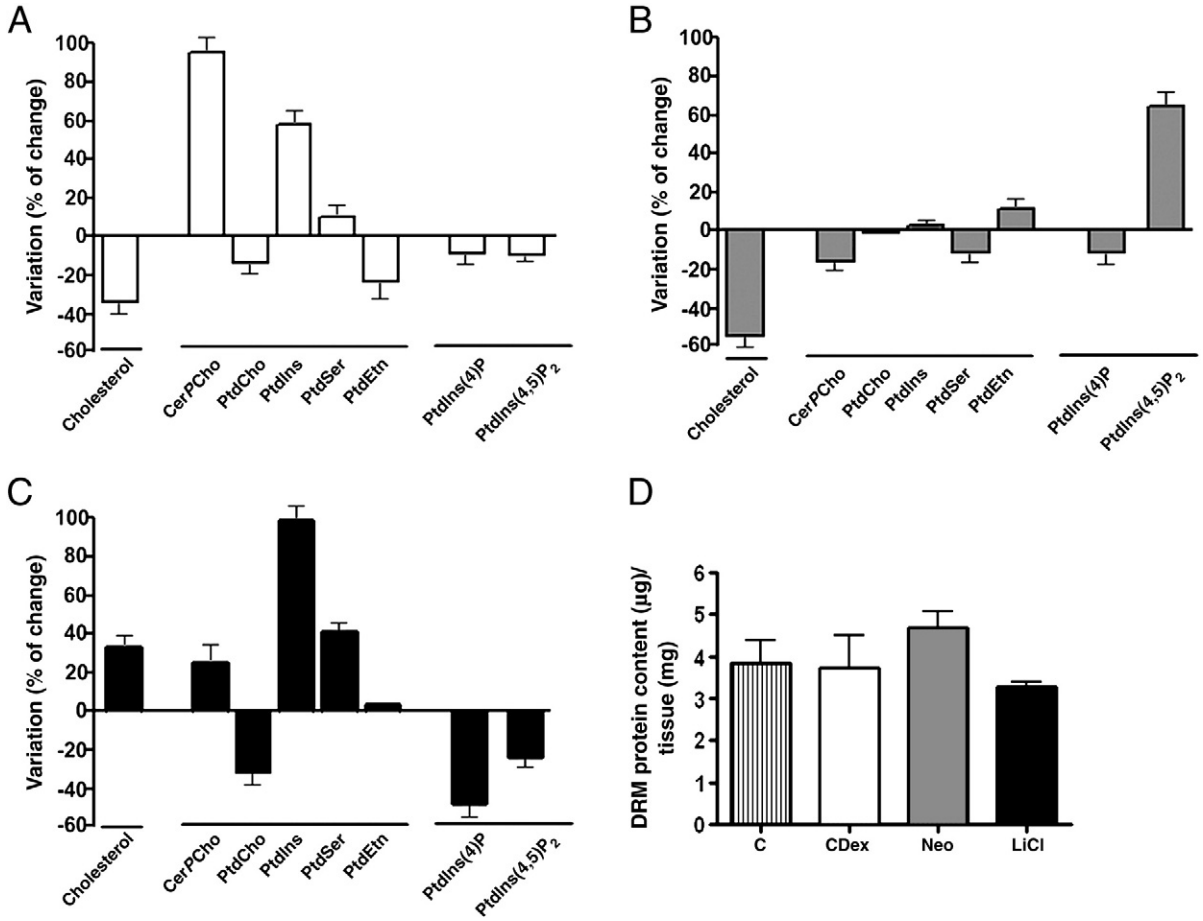

Fig. 3. Lipid composition and protein content of DRM after treatment with membrane-affecting agents. Renal papillary slices were treated with (A) 5 mM cyclodextrin, (B) 1 mM neomycin, or (C) 10 mM LiCl, and DRM were isolated. Variation in cholesterol, individual phospholipids, and polyphosphoinositides is expressed as percentage of control. (D) Variation in DRM protein content per mg of renal papilla tissue slices treated with 5 mM cyclodextrin (CDex) , 1 mM neomycin (Neo), or 10 mM LiCl (Mean ± SE, n = 5).

Both PtdIns(4)P and PtdIns(4,5)P_2 contents were diminished, accounting for a 50% and 25% decrease, respectively (Fig. 3C). Respect to the total protein level in the DRM fraction, no significant changes were observed (Fig. 3D).No changes in lipid composition of total cellular membrane were observed after the treatment with the various drugs (data not shown).

Taken together, the results demonstrate that the various membrane lipid-affecting agents caused a redistribution of membrane lipids, bringing about the formation of modified domains of different lipid composition, but did not alter the total DRM protein level.

Thereafter, we studied the presence of epithelial cell–cell adhesion proteins in total homogenates and in DRM isolated from microsomal fraction either treated or not with the different lipid-affecting agents by immunoblotting.

CD caused a slight decrease in DRM–E-cadherin content, whereas Neo caused a 50% decrease (Fig. 4A). The level of E-cadherin in total homogenate was significantly altered after CD treatment, whereas no significant changes were observed after Neo and LiCl treatments (Fig. 5A). These results demonstrate that despite the decrease in total amount of E-cadherin induced by CD, the protein persists in the modified DRM. By contrast, Neo affected mainly the DRM–E-cadherin while the total amount decreased by only 20%. On the other hand, the total level as well as the DRM content of the protein was resistant to LiCl treatment (Figs. 4A and 5A).

β-Catenin–DRM was profoundly affected by CD and Neo, showing 50% and 70% decrease in its content respectively, whereas no changes were observed by the treatment with LiCl (Fig. 4B). No significant changes in the amount of β-catenin were observed after any treatment in total homogenate (Fig. 5B). Consequently the low level of β-catenin in DRM was not due to the decrease in total level but to dissipation from DRM.

All three agents affected DRM–α-catenin content, since a 40% decrease was observed after CD and Neo treatments and a 20% decrease was observed after LiCl treatment (Fig. 4C). In total homogenate, CD and Neo did not significantly alter the total level of α-catenin, whereas LiCl caused a 25% decrease in its content (Fig. 5C). These results may indicate that while CD and Neo specifically dissipated α-catenin from DRM, the decrease induced by LiCl could be due to the decrease in total content.

E-cadherin forms complexes with catenins, which in turn, functionally link E-cadherin to the actin cytoskeleton [1]. Changes in the composition of the AJ complexes, and alterations in their interaction with the cytoskeleton have been suggested to play a key role in the regulation of cell–cell adhesion [2]. To analyze whether changes in the lipid composition of DRM affects the composition of the AJ complexes, we performed immunoprecipitation experiments to analyze the association of β-catenin with E-cadherin, as well as that of β-catenin with α-catenin. The immunoblot analysis showed a lower

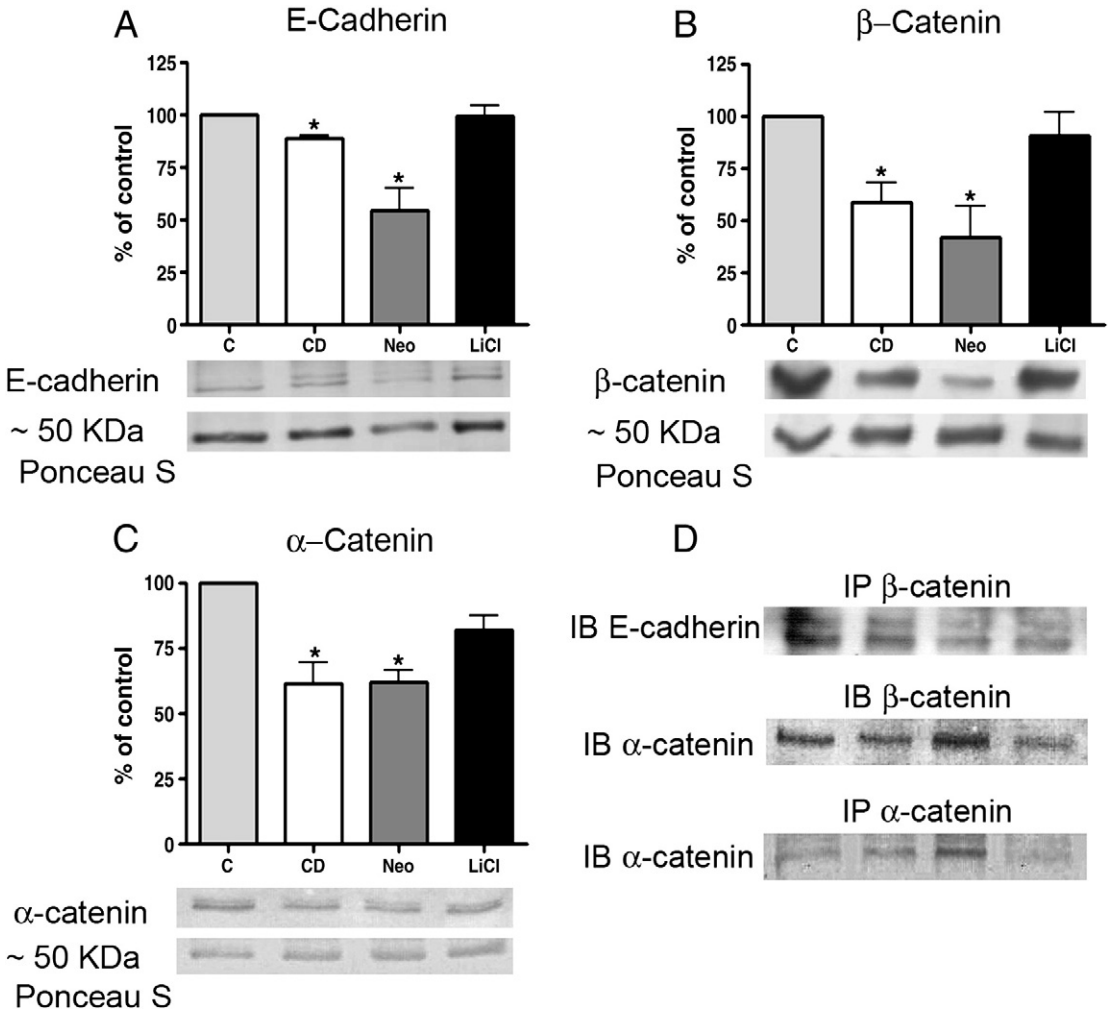

Fig. 4. Effect of changes in DRM lipid composition on the amount of E-cadherin (A), β-catenin (B) and α-catenin (C). DRM from microsome samples were obtained as described in Materials and methods, and equal amounts of total protein were developed in an 8% SDS-PAGE and analyzed by Western blotting. Protein loading was controlled by staining with Ponceau S. (Mean ± SE, n = 3). Representative immunoblots are shown. *Significantly different from control, $p<0.05$. (D) DRM were immunoprecipitated (IP) for β-catenin, or for α-catenin and then immunoblotted (IB) with the indicated antibody. Results correspond to a representative experiment of three individual assays.

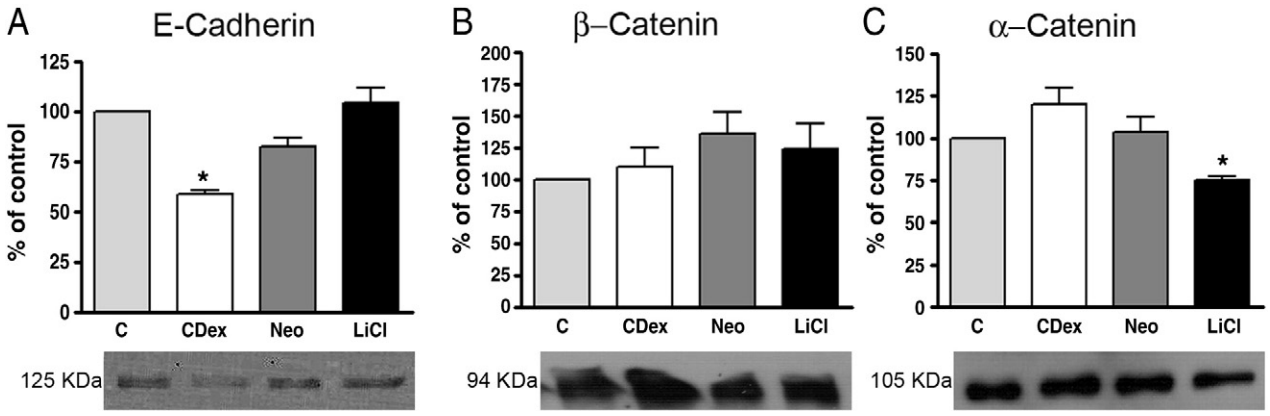

Fig. 5. Analysis of the total level of (A) E-cadherin, (B) β-catenin, and (C) α-catenin in renal papilla homogenates. Renal papillary slices were treated with 5 mM cyclodextrin (CDex), 1 mM neomycin (Neo), or 10 mM LiCl, and homogenate samples were obtained. Equal amounts of total protein were developed in an 8% SDS-PAGE and analyzed by Western blotting. Representative immunoblots are shown. (Mean ± SE, n = 3). *Significantly different from control, $p < 0.05$.

amount of E-cadherin in the precipitate of DRM isolated from cells treated with CD, Neo or LiCl, when a specific antibody anti-β-catenin was used (Fig. 4D). The amount of α-catenin that co-immunoprecipitated with β-catenin after CD and LiCl did not change, whereas the band obtained after Neo treatment increased (Fig. 4D). Similar results were obtained with β-catenin when the immunoprecipitation was performed with an anti-α-catenin antibody (Fig. 4D).

Since cholesterol is known to play an important role in lipid rafts, we further investigated whether the inhibition of cholesterol synthesis affected AJ proteins. To this end, we performed incubation with 25 μM lovastatin (Lova), an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, for 2 h. This treatment profoundly affected DRM–AJ protein association, since E-cadherin contents decreased by 70%, and β- and α-catenin contents decreased by 50% (Fig. 6A), but no change in the total protein level in DRM after lovastatin was observed (DRM protein content (μg/mg tissue) Control: 3.81 ± 0.66 vs Lovastatin: 3.71 ± 0.81, pNS). In total homogenates, the decrease in E-cadherin and α-catenin accounted only for 25%, and no decrease was observed for β-catenin (Fig. 6B).

Taken together, the results demonstrate that the various membrane lipid-affecting agents differently disturbed the interaction of the AJ proteins in DRM, thus causing a decreased assemblage of the AJ protein complexes.

3.3. AJ integrity depends on membrane lipid composition

In order to obtain a morphological correlation of the biochemical findings, we performed primary cultures of renal collecting duct cells and studied the effect of the membrane-affecting agents on AJ integrity. The fixed cultured cells were stained with anti-E-cadherin, anti-β-catenin, and anti-α-catenin. The protein distribution was visualized with confocal microscopy. In untreated cells, the phase contrast micrograph shows the typical morphology of cultured collecting duct cell displaying a well spread morphology (Fig. 7A). As shown in FITC-phalloidin stained cells, actin appeared organized as stress fibers and also as cortical network showing a correct cell organization of primary cultured cells, resembling tissue conformation (Fig. 7B). Within 30 min of CD treatment, the cells were more dispersed (Fig. 7C), and the phalloidin immunofluorescence showed disorganization of cell cultures, with loss of the epithelial morphology (Fig. 7D). In the center of the colony, cells appeared retracted and rounded, with the presence of cortical actin-based structures (Fig. 7D, arrowhead). Neo treatment caused a complete disintegration of cell–cell adhesion and a large percentage of cells appeared isolated (Fig. 7E). The phalloidin signal shows a colony in a process of disorganization (Fig. 7F). The loss of epithelial morphology, resembling that of the migrating cell, is clearly seen in a cell of the periphery of the colony (Fig. 7F, arrowhead). In LiCl-treated cells, the phalloidin images show an increased number of short stress fibers (Fig. 7H). The preservation of the actin organization in stress fibers can explain the maintenance of the cellular morphology (Fig. 7G). Lovastatin treatment caused a dissipation of cell–cell adhesions, the cells looked more dispersed (Fig. 7I), and, as shown in the phalloidin immunofluorescence, a great percentage of cells lost the epithelial morphology (Fig. 7J). It is accepted that the integrity of the AJ complexes is critical to the maintenance of stable adhesions [3]. To analyze whether changes in the lipid composition of DRM affect the interaction of these proteins at

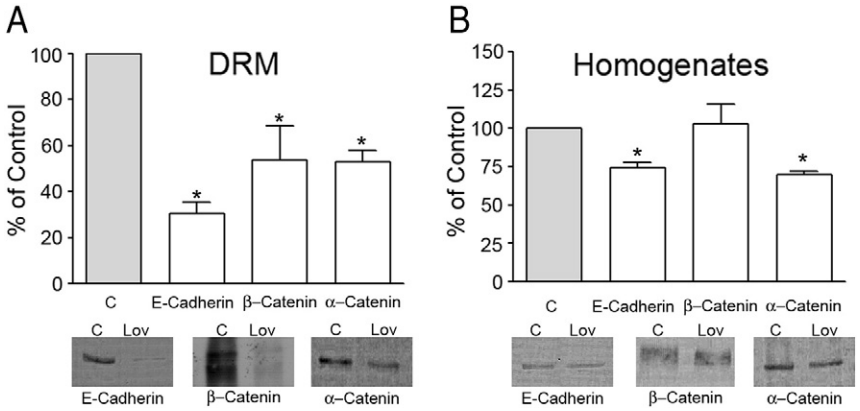

Fig. 6. Effect of lovastatin on the amount of E-cadherin, β-catenin and α-catenin content in (A) DRM and in (B) homogenates from renal papilla. Renal papillary slices were treated with 25 μM lovastatin. DRM or homogenates were obtained as described in Materials and methods, and equal amounts of total protein were developed in an 8% SDS-PAGE and analyzed by Western blotting. Representative immunoblots are shown. (Mean ± SE, n = 3). *Significantly different from control, $p < 0.05$.

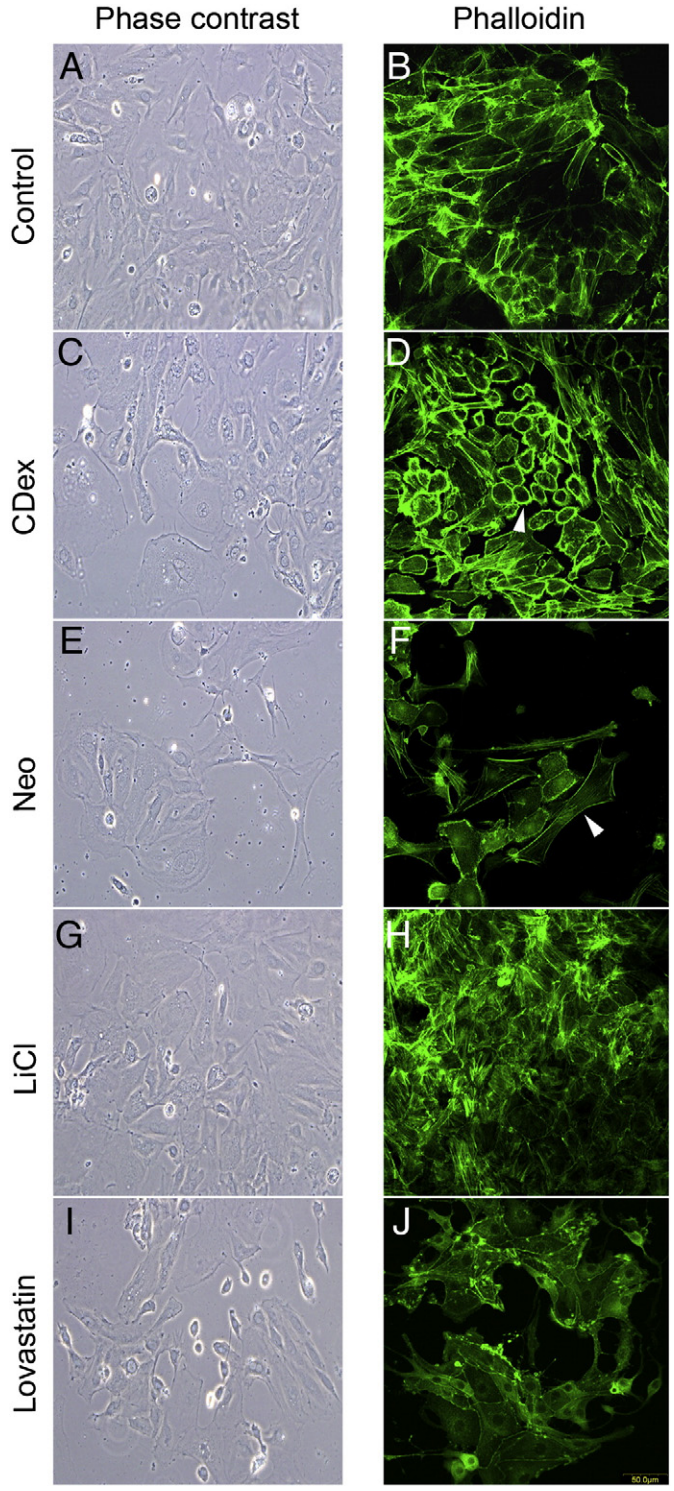

Fig. 7. Effect of lipid-affecting agents on the morphology and actin cytoskeleton of cultured collecting duct cells. Cultured collecting duct cells were treated with 5 mM cyclodextrin (C, D), 1 mM neomycin (E,F), 10 mM LiCl (G,H), or 25 μM lovastatin (I,J). After fixation, cells were observed with a phase-contrast microscope, or immunostained with FITC-phalloidin and observed with a confocal microscopy, and sections crossing the cell–cell contacts are shown. Representative images of three independent experiments are shown. Scale bars: 50 μm.

sites of cell–cell adhesions, we further studied cell–cell adhesion structures performing immunofluorescence microscopy using E-cadherin, β-, and α-catenin antibodies. We first studied E-cadherin and β-catenin distribution. The colocalization of AJ proteins was analyzed by using the Image Pro colocalization module. In untreated cells, E-cadherin as well as β-catenin lined the lateral membrane, and colocalized at the cell–cell contacts, thus reflecting the presence of AJ structures (Figs. 8A and B), as assessed in the segmentation images where colocalization appeared white (Fig. 8C). This is better observed in the enlarged figure inserts of cell–cell adhesion regions (Fig. 8C, insert). By contrast to the high degree of E-cadherin–β-catenin colocalization in untreated cells, each of the membrane-affecting agents induced an almost complete disappearance of colocalization between these proteins at cell–cell junctions, as seen in the representative segmentation images. After 30 min of CD treatment, the cell contacts appeared disintegrated and most of the E-cadherin dissipated from the lateral membrane (Fig. 8D), and appeared massively internalized as E-cadherin-containing vesicle-like structures that did not contain β-catenin (Fig. 8F, insert). By contrast, the β-catenin-positive signal is peripheric (Fig. 8F, insert) and presents β-catenin-positive but E-cadherin-negative bridges between adjacent cells (Fig. 8F, arrowhead). Neo treatment caused even more profound alterations, since cells were completely separated. Although E-cadherin as well as β-catenin distributed in the periphery of the cell, they did not form AJs (Figs. 8G and H). In a complete isolated cell, E-cadherin appeared delocalized from the plasma membrane and intensively accumulated in the cytosol (Fig. 8G, arrowhead). β-catenin distribution was similar to that observed for E-cadherin, with accumulation in certain zones of the cell (Fig. 8H, arrowhead). In non-rounded cells, although both E-cadherin and β-catenin were peripherically distributed, they did not colocalize, since only scarce points of overlapping were observed (Fig. 8I). In an isolated rounded cell, internalized vesicle-like structures were observed. Some were E-cadherin- and others β-catenin-positive, whereas only a few of them presented overlapping (Fig. 8I, insert). Interestingly, large β-catenin-positive and short E-cadherin-positive filopodia-like structures that did not overlap were also present (Fig. 8I, arrowhead). The different cell shape observed in the image Figs. 8G, H and I could reflect different stages of Neo deleterious effect. In LiCl-treated cells, no overlapping between AJ proteins was observed (Fig. 8L). Under LiCl treatment, cells still appeared spread but lost their cell–cell adhesion, conserving some β-catenin-stained bridges of connections between cells (Fig. 8K, arrowhead). While β-catenin still remained lining the cell periphery, E-cadherin was completely internalized, showing a punctuated pattern of distribution (Fig. 8J). In the magnified image (Fig. 8L, insert), it can be observed that cell connecting bridges are β-catenin- and E-cadherin-stained but appear as separated entities that did not colocalize. Lovastatin caused a massive E-cadherin internalization, with complete dissipation from plasma membrane localization, acquiring a punctuated pattern of distribution (Fig. 8M). By contrast, β-catenin still remained lining the cell, conserving some segments of cell–cell adhesion, but most of the cells are in the process of separation. In the magnified image of part of the cells, it can be observed that β-catenin is lining the periphery and E-cadherin as vesicle-like structures which did not contain β-catenin (Fig. 8O, insert).

Since besides interacting with the cytoplasmic domain of E-cadherin, β-catenin binds intracellular α-catenin to conform AJ, we further studied the β- and α-catenin distribution and their relationship (Fig. 9). In control, untreated cell cultures, β- and α-catenin had almost the same pattern of distribution, with a high degree of colocalization in the plasma membrane (Figs. 9A, B and C). After CD treatment, β-catenin as well as α-catenin mostly occupied the cell periphery, presenting zones of protein accumulation (Figs. 9D and E, arrowheads). Cell–cell adhesion was impaired and most of the cells started to separate. Some long bridges connected the cells and in the magnified image of a bridge it is possible to observe that both β- and α-catenin staining is present (Fig. 9F, insert). The images of β- and α-catenin colocalization show that although both proteins colocalized partially, cell–cell adhesion was not conserved (Fig. 9F). Neo caused the delocalization of both β- and α-catenins from the plasma membrane (Figs. 9G and H), but a thin line of non-interacting β- and α-catenins retained the rounded cells interconnected (Fig. 9I, arrowhead). Two polarized population of vesicles were also observed. One pole appears

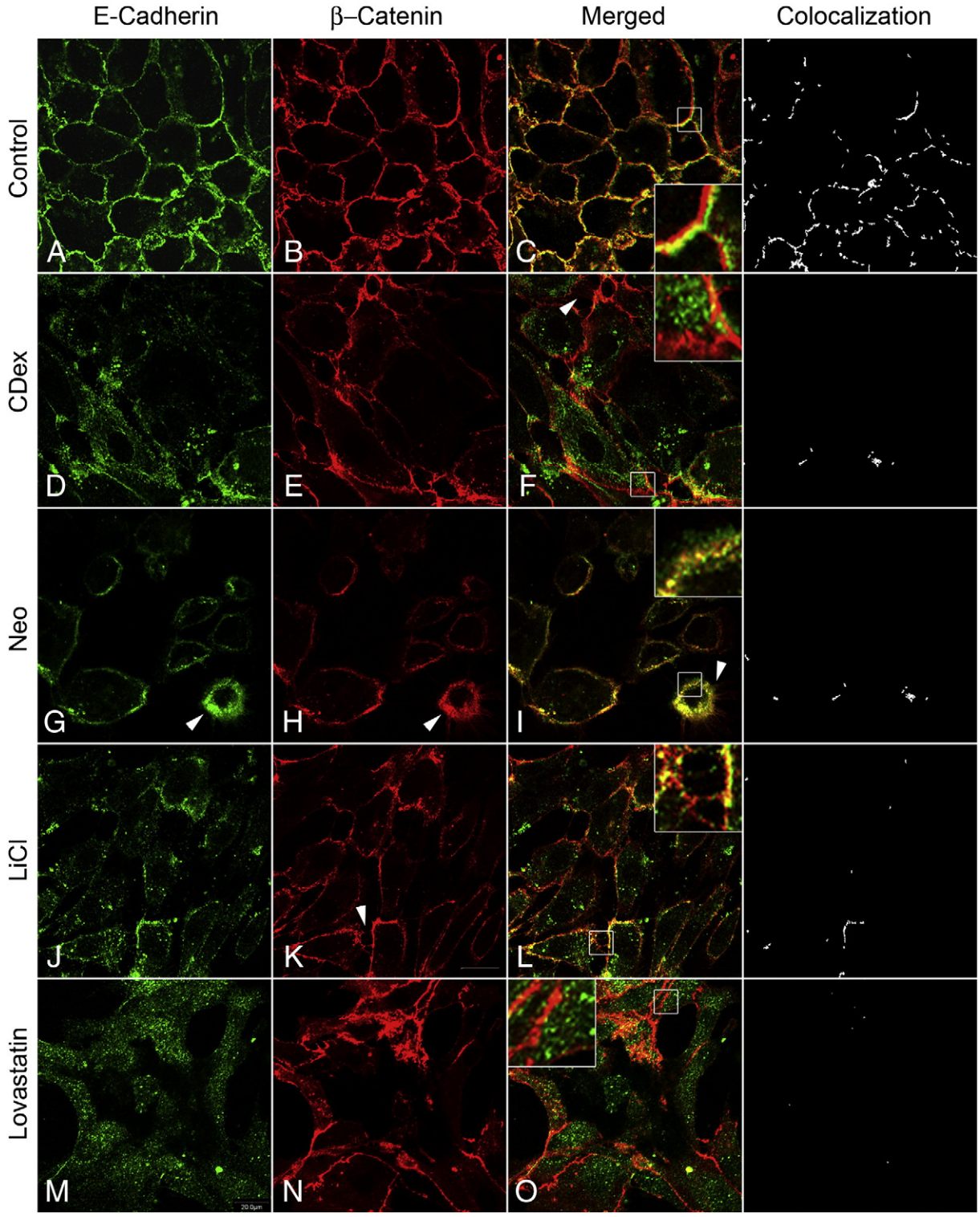

Fig. 8. Effect of changes in DRM lipid composition in the interaction of AJ proteins. Cultured collecting duct cells were treated with 5 mM cyclodextrin (CDex), 1 mM neomycin (Neo), 10 mM LiCl, or 25 μM lovastatin. After fixation, cells were simultaneously immunostained with antibodies against E-cadherin (green) and β-catenin (red). The colocalization study between E-cadherin and β-catenin was performed using the Image Pro colocalization module. Confocal sections crossing the cell–cell contacts are shown. Representative images of three independent experiments are shown. Scale bar: 20 μm.

enriched in α-catenin-containing vesicles, whereas the opposite pole appears enriched in β-catenin-positive vesicles (Fig. 9I, insert). After LiCl treatment, both β- and α-catenin remained partially in the periphery of the cells (Figs. 9J and K) but also an intracellular punctuate labeling pattern was also observed, denoting some protein internalization to peripheral organelles (Figs. 9J and K). Interestingly, despite the similar β- and α-catenin distribution, the detailed observation of the merged images shows that α-catenin appeared more externally localized than β-catenin (Fig. 9L, arrowhead), which justified the difficult cell–cell adhesion. By contrast, in the portion of the cell membrane where cell–cell adhesion persisted, β- and α-catenin colocalized (Fig. 9L, insert), as assessed in the segmentation image. Lovastatin

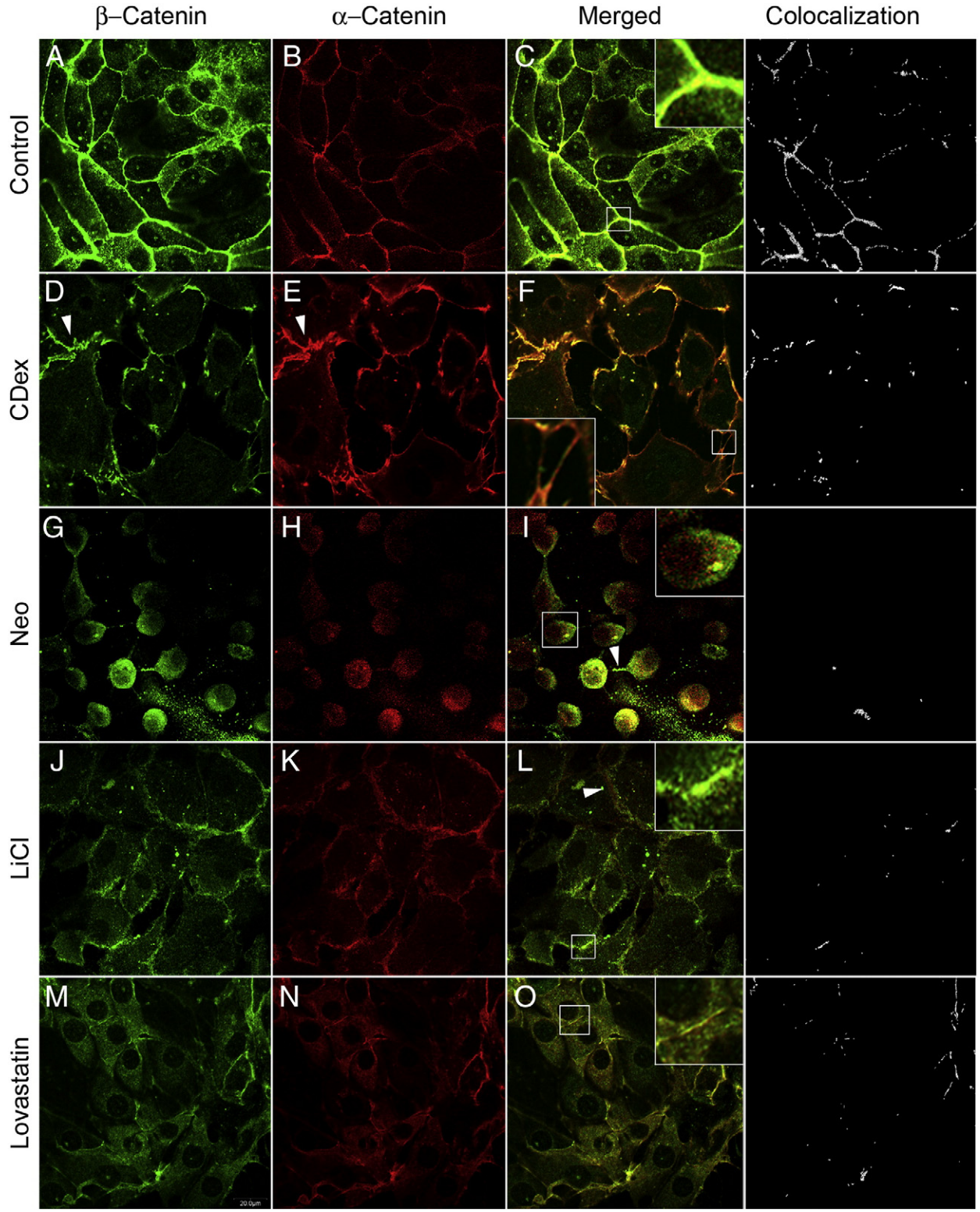

Fig. 9. Effect of changes in DRM lipid composition on the interaction of AJ proteins Cultured collecting duct cells were treated with 5 mM cyclodextrin (CDex), 1 mM neomycin (Neo), 10 mM LiCl, or 25 μM lovastatin. After fixation, cells were simultaneously immunostained with antibodies against β-catenin (green) and α-catenin (red). The colocalization study between β-catenin and α-catenin was performed using the Image Pro colocalization module. Confocal sections crossing the cell–cell contacts are shown. Representative images of three independent experiments are shown. Scale bar: 20 μm.

treatment induced internalization of β- as well as α-catenin, with similar intracellular distribution. However, there are some zones of the membrane where both catenins persisted and colocalized maintaining zones of cell–cell adhesion (Fig. 9O, insert). By contrast, some internalized vesicle-like structures are β-catenin and others are α-catenin-positive.

4. Discussion

In the present work, we studied the importance of membrane lipid composition in the maintenance of E-cadherin-mediated cell–cell adhesion. To this end, we took advantage of the fact that cultured

papillary collecting duct cells preserve their tendency to interact with their neighboring cells, mimicking their behavior in intact tissue. Therefore, we established a parallelism between the biochemical data obtained from renal papillary collecting duct microsomes and the morphological observations from immunofluorescence microscopy performed in primary cultures of collecting duct cells. By combining biochemical and immunofluorescence studies, we demonstrated that the membrane lipid composition affects the in vivo preservation of AJ.

We first demonstrated that the AJ complexes were present in DRM. It has been demonstrated that the DRM composition critically depends on the cell type, the starting material and the isolation protocols [27]. For this reason, we analyzed the lipid composition of the isolated DRM and found that they had the biochemical characteristics of lipid rafts. Besides the enrichment in cholesterol and sphingomyelin, the isolated DRM are also enriched in PtdIns(4)P and PtdIns(4,5)P_2, consistent with the earlier observation of Hope and Pike [28]. In accordance with our results, previous studies performed in MDCK cells [14], a cell line of dog kidney collecting ducts, and in murine fibroblasts [29] have shown the association of the AJ proteins with the Triton X-100 insoluble fraction. More recently, immunofluorescence and biochemical experiments demonstrated that N-cadherin of AJ colocalized with lipid rafts in C2C12 myoblasts [30]. In our opinion, the above findings highlight the relevance of rafts as membrane platforms where cadherin-mediated cell adhesions are located. It has been proposed that because of their relative stability and complexity, cell–cell junctions can be considered membrane microdomains themselves [15]. In the present work, we used various membrane-affecting agents as tools to study the influence of the membrane lipid composition on AJ preservation. We showed that such agents affected the AJ components differently, depending on their capacity to change the membrane lipid composition. In accordance with previous observations in smooth muscle cells [31], cyclodextrin did not lead to raft elimination. Instead, here we showed that besides inducing a cholesterol decrease, cyclodextrin caused membrane lipid redistribution, leading to a modified microdomain with lower cholesterol but higher sphingomyelin content. With respect to cell–cell adhesion, the immunofluorescence images show that cyclodextrin caused an almost complete separation of collecting duct cells. This fact, which is not compatible with epithelial tissue preservation, seems to be due to an impairment of E-cadherin span in the membrane domain of new lipid composition. It appears that in such environment E-cadherin internalized rather than stayed in the plasma membrane. As E-cadherin internalized, AJ dissipated. Dissipation of AJ is consistent with the decrease in AJ complexes in DRM, which is also supported by the decreased content of β- and α-catenins as observed by inmunoblotting. However, DRM–E-cadherin was not very significantly lower in CD-treated than in control DRM, possibly due to vesicular E-cadherin that co-sedimented with membrane DRM during the process of isolation. Thus, we suggest that CD induces modifications in the lipid composition, creating a different lipid environment where E-cadherin internalizes and where β- and α-catenins are excluded, thus causing AJ dissipation.

Neomycin appeared as the most deleterious agent, as denoted in the phalloidin-stained images. Consistently, AJ proteins dissipated from DRM, as observed by immunoblotting. The significant decrease in cholesterol, together with the absence of the counterbalance increase in sphingomyelin, may cause an extremely non-favorable lipid domain for the AJ proteins. It is known that cholesterol has high affinity for sphingomyelin and probably the decrease of cholesterol is secondary to the sphingomyelin fall. Interestingly, although in lower amounts, E-cadherin persisted in the plasma membrane, and partially colocalized with β-catenin, whereas α-catenin appeared as the most affected AJ protein. Recently, Weiss and Nelson [32] have reported that the α-catenin interaction with β-catenin and that with the actin filament exclude mutually. Interestingly, we here showed that, although AJ complexes were impaired by Neo, an increase in the amount of α-catenin bound to β-catenin was obtained. Such an increase in the β-catenin–α-catenin interaction may affect the interaction of the latter with actin filaments. Thus, the action of Neo appeared dividing β-catenin in two pools: one that persists bound to E-cadherin and the other that is pulled in by α-catenin. Probably, by increasing the interaction between β-catenin and α-catenin, the α-catenin–actin filament binding diminishes, thus producing the overall alteration of the epithelial cell morphology.

Taking into account that cultured cells were treated with cyclodextrin and neomycin for a short period of time (30 and 10 min, respectively), it is evident that the membrane domains where AJ complexes are located are highly sensitive to the effect of such agents. Similar results were obtained when cells were treated with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase that decreases intracellular cholesterol concentration, thus corroborating that cholesterol is crucial to maintain AJ structure.

LiCl also caused the impairment of AJ. E-cadherin internalized whereas β and α-catenins persisted in the plasma membrane. Although both β- and α-catenins remained localized in the plasma membrane, they did not colocalize. Consistently, the treatment with LiCl is the condition where less α-catenin associates with β-catenin, as is seen in the immunoblot. Both findings reflected failure in the interaction between β- and α-catenin. Interestingly, LiCl caused the appearance of a huge number of stress fibers which is consistent with our previous observation that LiCl induces an increase of talin containing focal adhesions [16]. LiCl caused no loss of cholesterol and sphingomyelin but changed the relative proportion of neutral and acidic phospholipids, and in such environment AJ were impaired. Thereafter, it is evident that the maintenance of the overall lipid composition of AJ domains is critical to maintain the integrity of AJ. These results allow us to hypothesize that transient, physiological changes in the phospholipid profile of AJ domains might physiologically modulate AJ turnover. We consider that the various agents used change specifically the lipid composition of DRM fraction because in DRM are concentrated the phospholipid hydrolysing enzymes and also the phosphoinositides kinases thus regulating the local lipid composition. It is accepted that membrane sphingomyelin is an endogenous inhibitor of phospholipases. We consider that the changes in sphingomyelin content can induce the activation of local phospholipases, thus affecting the local concentration of phospholipids.

It is accepted that the continuous expression and functional activity of E-cadherin are required for cells to remain tightly associated in the epithelium, and that, in their absence; the other cell-adhesion proteins are not capable of supporting intercellular adhesions [33]. Both during tumorigenesis and normal development, epithelial cells lose intercellular adhesions. This has been attributed to low levels or absence of E-cadherin expression [33,34]. Here we report loss of AJ caused by modification of the lipid membrane environment where cell-adhesion proteins assemble to form AJ complexes. This work constitutes the first report of cell–cell adhesion impairment due to changes in their lipid environment.

It is interest to point out that both neomycin and LiCl are pharmacological agents of known nephrotoxic effects. In this context, our results could also be pharmacologically relevant. Neomycin is an aminoglycoside antibiotic known to cause tubular necrosis [35,36]. Here we showed that a short period of neomycin treatment is able to destroy cell–cell adhesions. The disruption of cell–cell adhesions could explain the deleterious effect caused by neomycin treatment. Regarding LiCl, it is chronically used for treatment of some human mental diseases [37,38] and it is known that long-term treatment with this agent causes alterations in the renal capacity for concentrating urine [39]. It is known that the papillary collecting duct is in fact the structure where the final adjustment of urine volume occurs. Although the pharmacological dose of LiCl is lower (0.8–1 mM) than the concentration used here, we hypothesize that chronic low doses of LiCl could also impair cell–cell adhesion and thus affect renal function. Lovastatin is used as a pharmacological agent to treat hypercholesterolemia.

Although, to our knowledge, no nephrotoxic effect has been reported, our results suggest the possibility that it can induce deleterious effects on epithelial renal tissues.

In the present report we present evidence on the involvement of the lipid membrane composition in AJ integrity and suggest that the DRM isolated in our experimental conditions is a submicrodomain that serves as a platform to localize AJ components and that behaves as a biochemical unit facilitating the correct interaction of cell–cell adhesion components. In addition, we propose that the maintenance of the physiological lipid composition of DRM is a requisite to preserve cell–cell adhesion and consequently the collecting duct tubular organization and its functionality.

5. Conclusions

We used various membrane-affecting agents as tools to study the influence of the membrane lipid composition on AJ preservation on collecting duct cells. Methyl-β-cyclodextrin does not lead to raft elimination. Instead, provokes membrane lipid redistribution, leading to a new membrane domain with lower cholesterol but higher sphingomyelin content. In such environment E-cadherin internalizes, and β- and α-catenins are excluded, thus causing AJ dissipation. Neomycin induce a decrease in cholesterol with the absence of the counterbalance increase of sphingomyelin, causing an extremely non-favorable lipid domain for the AJ proteins as denoted by the loss of cell–cell adhesion and the alteration of the epithelial cell morphology. The decrease in cholesterol is a common feature in the deleterious effect of cyclodextrin and neomycin. We thus suggest that cholesterol is crucial for bringing out the lipid environment necessary to maintain the AJ complexes assembled. The use of lovastatin, another cholesterol modifier agent, corroborates this statement. LiCl causes no loss of cholesterol and sphingomyelin but changes the relative proportion of neutral and acidic phospholipids, and in such environment AJs are impaired. The maintenance of the overall lipid composition of AJ domains is critical to maintain the integrity of AJs. These results allow us to hypothesize that transient, physiological changes in the phospholipid profile of AJ domains might physiologically modulate AJ turnover. This work constitutes the first report of cell–cell adhesion impairment due to changes in their lipid environment. It is interest to point out that both neomycin and LiCl are pharmacological agents of known nephrotoxic effects. In this context, our results could also be pharmacologically relevant.

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