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$_3$VO$_4$. 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$_3$VO$_4$. 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 sediment 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.