Endocytosis of Junctional Cadherins in Bovine Kidney Epithelial (MDBK) Cells Cultured in Low Ca\textsuperscript{2+} Ion Medium

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Abstract. The release of intercellular contacts in MDBK cells, initiated by the depletion of Ca\textsuperscript{2+} ions from the culture medium, results in the endocytotic uptake of membrane vesicles containing specific membrane constituents of the zonula adhaerens (ZA). During this process the junction-derived, endocytosed vesicles remain associated with the ZA plaque components, while the plaque and its attached actin filaments retract as a whole in a ring-like fashion from the plasma membrane, often accumulating, usually in fragments, in the juxtanuclear cytoplasm. Double-label immunofluorescence microscopy with anti-plakoglobin and antivinculin has indicated that both plaque proteins colocalize with the hallmark membrane glycoprotein of this junction type, E-cadherin (uvomorulin). When HRP used as a fluid phase marker is applied to the culture medium, simultaneously with the Ca\textsuperscript{2+} ion-chelator EGTA, numerous HRP-positive vesicles are found in close association with the dislocated plaque material, suggesting that the HRP is contained in the vesicles formed upon EGTA-induced junction splitting. Immunoelectron microscopy with various cadherin-specific antibodies revealed vesicle-associated labeling, confirming the derivation of these plaque-associated vesicles from the ZA. As the desmosome-specific cadherin, desmoglein, is recovered in another type of junction-derived vesicle, which is characterized by its association with a desmoplakin-plaque, we conclude that the membrane domains of both kinds of junction are endocytosed during Ca\textsuperscript{2+} depletion but stay in different vesicle populations, emphasizing the selective interaction of the specific cadherins with their respective plaque and filament partners.

The apical region of polarized epithelial cells contains two types of cytoskeletal filament-anchoring junctions, i.e., the desmosomes (maculae adhaerentes) connected to intermediate-sized filaments (IFs)\textsuperscript{1} and the actin filament-associated zonula adhaerens (ZA), both contributing to cell-to-cell adhesion as well as to intracellular organization (for reviews see 15, 26, 27, 49, 50). The two kinds of junctions are distinct by biochemical and morphological criteria, yet they share some basic architectural principles and have at least one major constituent protein in common: their junctional membrane domain is intimately associated with plakoglobin (82 kD; 10, 19, 20, 36).

In both types of junctions, the direct mediators of cell-to-cell adhesion appear to be transmembrane glycoproteins that are concentrated, or exclusively located, in the specific junction membrane domains, and those that have been characterized so far belong to the cadherin superfamily of cell adhesion molecules: desmoglein and desmocollins I and II are localized in desmosomes (4–7, 29, 30, 35, 39, 47–49, 51), while the major cell adhesion molecule of the ZA is E-cadherin (uvomorulin, L-CAM; 2, 3, 14, 22, 33, 46, 53, 58).

It has also been shown that the cytoplasmic domains of the desmosomal glycoproteins and of the ZA-cadherin(s) contribute to the formation of the specific filament-anchoring cytoplasmic plaques (11, 33, 43, 44, 47, 48, 51). In addition to the COOH-terminal, cytoplasmic portions of their specific cadherins, these plaques contain, besides plakoglobin, a set of cytoplasmic proteins that are characteristic of either desmosomes or the ZA: in polarized epithelial cells, desmosomes have been shown to contain desmoplakin (5, 8, 9, 17–19, 32, 42, 49), whereas vinculin has been localized to ZA plaques (23–27; see 12 and 54 for other ZA proteins). Cadherins are Ca\textsuperscript{2+}-binding proteins, and cadherin-mediated cell-to-cell adhesion is dependent on a certain concentration of extracellular Ca\textsuperscript{2+} (for review see 53). In previous studies it has been shown that diverse types of cultured polar epithelial cells dissociate upon removal of Ca\textsuperscript{2+} ions from the culture medium. During this process, half-desmosome equivalents are internalized in the form of endocytic vesicles, including some desmosomal transmembrane glycoproteins, in association with the cytoplasmic plaque material and associated IF bundles (8, 13, 37, 38, 41, 48). The splitting of these cell-to-cell junctions in response to Ca\textsuperscript{2+} depletion also results in the release of ZA plaque material from the plasma membrane: ring-like structures containing vincu-

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1. Abbreviations used in this paper: IF, intermediate-sized filament; ZA, zonula adhaerens.
lin and actin have been identified deep in the cytoplasm, retracting centripetally to a juxtanuclear position (37, 57). However, the fate of the corresponding junctional membrane components, notably the cadherins, remained unresolved.

In our previous study of Ca2+-depleted MDBK cell cultures (57) we have not recognized endocytotic ZA-derived membrane structures. More recently, however, we have applied several antibodies to ZA-specific cadherin(s), together with immunoelectron microscopy, to identify certain plasma membrane invaginations and vesicles containing fluid phase markers, and we can now show that during Ca2+ depletion ZA membrane is also resumed into the cell interior, forming ZA plaque-associated vesicles. Therefore, we conclude that both kinds of junctions behave in a principally similar way, i.e., their membrane domains endocytose, but are kept separately, probably due to their remaining attachment to different filament systems.

Materials and Methods

Cell Culture

Bovine kidney epithelial cells of the line MDBK (ATCC, CCL 22; 40) were grown on glass cover slips in MEM with nonessential amino acids and Earle's basal salt solution, containing reduced bicarbonate concentration (0.85%) and 10% FCS. The calcium ion concentration of the medium was 1.8 mM. Cells were grown to ~80% of confluency and routinely used on the second or third day after plating.

Antibodies

The following mAbs have been used: antidesmoplakin I and II (2.15, 2.17; 9), antidesmoglein (DG 3;10; 47, 48), antiplaqueolin (PGS 1; 10), and antitivinuculin (VIN 11.5; 57). Rabbit antibodies to uromorulin (55, 56) were kindly provided by Dr. R. Kemier (Max-Planck-Institute for Immunology, Freiburg, FRG). Rabbit antibodies "anti-L-CAM" (R14; prepared as described, 3) recognizing a 120-kD polypeptide on MDBK cells were kindly provided by Dr. J. P. Thiery (Institute of Developmental Physiology, Ecole Normale Supérieure, Paris, France). Pan-cadherin rabbit antibodies (R156 and R157) raised against a synthetic peptide representing the conserved COOH-terminal sequence of N-cadherin, recognizing 135- and 120-kD polypeptides on MDBK cells, have been described (28). Clathrin antibodies (OD 9.5) were purchased from Progen Biotechnics (Heidelberg, FRG). Actin was visualized by using a phallolidin-rhodamine (tetramethyl-rhodamine isothiocyanide) conjugate (Sigma Chemical Co., St. Louis, MO). Appropriate secondary antibodies coupled to FITC or to Texas red were purchased from Dianova (Hamburg, FRG). Goat anti-rabbit (IgG plus IgM) antibodies coupled to 5-nm colloidal gold particles were from Janssen (Beerse, Belgium), and goat antirabbit peroxidase-conjugated F(ab')2 fragment antibodies for 30 min each. Removal of unbound antibodies with PBS after each step was followed by fixation with buffered 2.5% glutaraldehyde (0.05 M sodium cacodylate, pH 7.2, 50 mM KCl, 2.5 mM MgCl2) for 10 min, washing in buffer (as above), and transfer to the DAB reaction solution (31). For both procedures, fixation and processing for ultrathin sectioning were as mentioned above.

Results

Immunofluorescence Microscopic Detection of ZA

Immunofluorescence microscopy of densely grown MDBK cells with antibodies to components of the ZA plaque, such as vinculin and plakoglobin, as well as with antibodies to the extracellular portion of ZA-cadherin(s), revealed an essentially identical staining along cellular contact sites (Fig. 1, a, b, and d). The rabbit pan-cadherin antibodies (R156 and R157), which react with the cytoplasmic side of the molecule(s), also stained the MDBK cell boundaries (Fig. 1, c and e). This obvious colocalization of cytoplasmic plaque components with cadherin(s) at the light microscopic level was particularly clear from double-label experiments as shown in Fig. 1, a-d. The close vicinity of the ZA and the subapical ring of desmosomes is demonstrated in Fig. 1, e and f, using antibodies to E-cadherin and desmoplakin, respectively.

The morphology and the cellular distribution of the ZA markers changed dramatically after Ca2+ ion depletion from the culture medium. Within minutes after addition of EGTA, the cadherins, labeled with antivomirulin (Fig. 2 a) or pan-cadherin antibodies (Fig. 2, b, d, and f), appeared in ring-like cytoplasmic structures in most (80-90%) of the cells, apparently dislocated from the cell surface, and double-label experiments showed that the plaque components, plakoglobin and vinculin, largely colocalized in the same structures (e.g., Fig. 2, c and e). At later time points after the EGTA treatment (30-45 min), the diameters of these ZA-derived rings, still positive with the various marker antibodies described above, became smaller and more disrupted. Similar results were also obtained with the polyclonal antibodies of rabbit serum R14 (not shown). The diameter of these ring-like structures varied due to the general variation.
of the mean circumference of the MDBK cells and due to the fact that the formation of these structures was neither absolutely synchronous nor complete in all cells (see, e.g., Fig. 2 a, arrows). The rings then became less distinct and could hardly be detected after more than 1 h of incubation in the presence of EGTA (not shown). Accumulation of the endocytosed E-cadherin in larger vesicles indicative of lysosomal elements, was not observed. By direct comparison, in the same cells, the dissociation of the ZA and the dislocation of ZA plaque material from the cell periphery was always faster than the splitting and endocytotic uptake of the desmosomal components (Fig. 2, f and g). Double labeling for ZA-cadherin(s) and desmoplakin/desmoglein of cells treated for 5–10 min with EGTA showed, on the level of detection of the method used, no colocalization. While individual ZA-cadherin–positive ring-like or arc-like structures were found in
Figure 2. Immunofluorescence microscopy showing components of the ZA and of desmosomes in MDBK cells after Ca\(^{2+}\) ion depletion of the culture medium by addition of 4 mM EGTA. (a) Immunofluorescence microscopy with E-cadherin (uvomorulin) antibodies reveals ring- or arc-shaped structures in the cytoplasm (45 min treatment). Arrows point to areas of the ZA that have not yet detached completely from the cell surface. (b–e) Double-label immunofluorescence with pan-cadherin (b) and plakoglobin (c) antibodies, or pan-cadherin (d) and vinculin (e) antibodies. Note colocalization on ring-like structures (some are denoted by arrows) of transmembrane (b and d) with cytoplasmic plaque (c and e) components of the ZA 30 min after onset of treatment. (f and g) The effect of EGTA treatment (10 min) on the ZA and on desmosomes, as shown by double-label immunofluorescence with pan-cadherin (f) and desmoplakin (g) antibodies,
Figure 3. (a–d) MDBK cells fluorescently labeled for actin with rhodamine-phalloidin, to show the fate of the ZA-associated actin upon treatment with 4 mM EGTA for 0 (a), 15 (b), 60 (c), and 120 min (d). At early time points of the treatment, continuous “ring-like” structures are seen in the cytoplasm, which represent the centripetally retracted, ZA-associated microfilaments (b). These “actin belts” then break down gradually (c), leaving, in many cells, only fragmented substructures of the former actin belt (arrows in c and d). Besides the bright staining of the internalized ring structures, another (“secondary”) peripheral belt of actin filament material can be recognized (small arrows in c and d), which becomes increasingly apparent as the internalized primary ring-like structures break down. Bars: (a–d) 20 μm; (e) 1 μm.

Actin as another cytoplasmic ZA-attached component, visualized by labeling with rhodamine-coupled phalloidin, could also be localized, under fixation conditions chosen, to a continuous belt in untreated MDBK cells, representing the ZA-associated microfilaments (Fig. 3 a). This “actin belt” retracted centripetally after addition of 4 mM EGTA to the culture medium, and individual cytoplasmic ring-structures, identical to those seen with antibodies to vinculin, plakoglobin, and ZA-cadherin(s), became visible (Fig. 3 b), confirming our previous results (57). With the gradual breakdown of these ring structures, at ~60 min after EGTA addition (Fig. 3 c), and, finally, the disappearance of the retracted ZA actin material, another peripheral belt of actin filaments became increasingly visible at the cell periphery (Fig. 3 d). As the cells round up during EGTA treatment, this new actin-positive peripheral ring becomes more prominent, probably representing filamentous structures of the cell cortex. Double-label experiments with clathrin antibodies showed no colocalization with E-cadherin–positive vesicles appearing in the cytoplasm after EGTA treatment (not shown).

EM of Normal and HRP-EGTA–treated MDBK Cells
Cross sections of confluent MDBK cell monolayers revealed the subapical localization of the ZA in this cell type (Fig. 4 demonstrates that endocytosis of ZA components is faster than endocytosis of desmosomal halves. The arrowheads in f point to situations where individual ring-like structures have already clearly detached from the cell periphery (f), whereas the desmosomes (arrows in g) are still nearly unchanged. Bars, 20 μm.
The extended plaque structure (2–3 μm high and 0.5–0.8 μm thick) appeared as a circumferential belt of a bundle of microfilaments with interspersed clusters of electron-dense, amorphous material ("dense bodies"; Fig. 4a). Sections running tangentially to such belts show that these densities are arranged in an extended convoluted structure, located within the straight-aligned microfilament bundle (Fig. 4b). Treatment with EGTA caused the splitting of intercellular contacts, the release of the belt-forming plaque from the cell surface, and the appearance of ring-like structures in the juxtanuclear cytoplasm (not shown; see 37, 57). In addition to these dislocated microfilament bundles associated with ZA components, another large belt-like filamentous structure could be detected in the cortical cytoplasm of cells that had been treated with EGTA for at least 30 min. This "outer ring" became more prominent at later time points (e.g., 90 min; Fig. 4c), showed interspersed densities (see arrowheads in Fig. 4c), and revealed no associated membrane profiles. At such late time points, however, the dislocated ZA components had already lost their ring- or arc-like continuity and

Figure 4. Electron micrographs of MDBK cells, demonstrating the subapical localization of a cross (a) and tangentially (b) sectioned ZA. The extended plaque structure appears as a belt or bundle of microfilaments, with interspersed clusters of amorphous material of higher electron density (a, arrowheads), which in flat sections is revealed as a convoluted structure (b). (c) In cells treated with EGTA for 90 min, the dislocated ZA plaque structure can be found in a juxtanuclear position (large arrows), while another filamentous belt structure is now seen in the cortical cytoplasm (arrowheads point to interspersed densities). Note that vesicles are not associated with this "outer ring." N, cell nucleus; M, mitochondrion. Bars: (a and b) 0.5 μm; (c) 1 μm.
formed a fragmented substructure in the vicinity of the cell nucleus (Fig. 4 c, arrows; compare also with Fig. 3, c and d, arrows).

When MDBK cells were incubated in a medium containing 4 mM EGTA together with HRP (3–5 mg/ml) as a "liquid phase marker," the HRP reaction product was found trapped in vesicles of various sizes that were associated with the ZA plaques detached from the surface membrane. The majority of these ZA plaque-associated, HRP-positive vesicles were intimately associated with the electron-dense bodies mentioned above, whereas an attachment to actin filaments was not regularly noted (Fig. 5, a and b). In addition, some HRP-
Figure 6. Immunoelectron microscopy of MDBK cells incubated with E-cadherin (uvomorulin) antibodies at 5 (a), 15 (c), and 30 min (b and d) after addition of 4 mM EGTA to the culture medium. Bound antibodies were visualized by peroxidase-coupled secondary antibodies and enzyme reaction product. Before incubation with antibodies, cells were lysed with a low saponin concentration (0.01%) in PBS for 10 s (a and b), or with a higher saponin concentration (0.03%) in PBS for 30 s (c and d). These antibodies, known to react specifically with the extracellular portions of the molecules (3), could be localized either at the intermembranous surface of the ZA still unaffected by the EGTA treatment (a, arrowheads in c), or within vesicles found to be associated with the partially (a, arrows) or completely detached plaque material (b–d, arrows). In the pictures showing contrasted (a), half-contrasted (b and d), or uncontrasted (c) sections, the small arrowheads point to the dislocated plaque structure of the ZA. Brackets (c and d) indicate reaction sites still at the cell surface. The inset in a shows the lower left portion of micrograph (a, arrowhead) at higher magnification. N, cell nucleus; PM, plasma membrane. Bars: (a) 1 μm; (b, d, inset in c and d) 0.2 μm.
loaded vesicles of the “normal” endocytic pathway, such as coated vesicles, endosomes, and lysosomes, were observed in various positions of the cytoplasm also, but were never closely associated with the ZA plaque–derived structures (e.g., Fig. 5 a). When the cells were treated for 10 min with EGTA alone and HRP was added for a subsequent pulse of 20 or 30 min, the ZA plaque–associated vesicles, however, were free of HRP reaction products, whereas membrane compartments of various forms and sizes, representing the different components of the endosomal/lysosomal system, were heavily stained in this case (Fig. 5 c).

**Electron Microscopic Immunolocalization of ZA-Cadherins**

To characterize the fate of ZA components upon EGTA treatment in greater detail, we performed immunoelectron microscopy, using the same ZA-cadherin–specific antibodies.

Labeling with uvomorulin antibodies that specifically react with the extracellular moiety of the molecule (see reference 3) was seen along the ZA midplane in untreated cell monolayers, whereas junction-free areas of the lateral plasma membranes were not labeled or were only very sparsely labeled (data not shown). During the first minutes (2–5 min) after EGTA treatment, the plaque material began to detach from the cells and was found free of HRP reaction products, whereas membrane domains (e.g., Fig. 5 a). When the cells were treated for 10 min with EGTA alone and HRP was added for a subsequent pulse of 20 or 30 min, the ZA plaque–associated vesicles, however, were free of HRP reaction products, whereas membrane compartments of various forms and sizes, representing the different components of the endosomal/lysosomal system, were heavily stained in this case (Fig. 5 c).

Using different immunoelectron microscopic (immunogold and immunoperoxidase) procedures, we observed vesicles that were associated with ZA plaque material and contained trapped cadherin antibodies labeled by HRP (Fig. 6, a–d) or gold particles (Fig. 7, a and b). At these early time points, antibody staining was still also found at ZA cell contact sites yet unaffected by the treatment (Fig. 6, a and c). A much higher number of vesicles stained with the antiuvomorulin were seen, in association with the detached plaque material at later time points (e.g., 30 min; Fig. 6 b). Shape, frequency, and association of the immunoreactive vesicles with the dislocated plaque material were similar to those labeled with the fluid phase marker (compare, e.g., Fig. 5, a and b with Fig. 6, b and d).

Using pan-cadherin antibodies specifically reacting with the cytoplasmic portion of these molecules (28) we also found, in EGTA-treated cells, antibody label on the cytoplasmic surfaces of vesicles that were closely associated with the dislocated plaque (Fig. 7 c) or in close vicinity of the plaque structure (Fig. 7 d). Due to the more stringent experimental conditions needed for the penetration of the gold-conjugated secondary antibodies, the membrane profiles of the vesicles were occasionally less well-preserved (Fig. 7 d). However, gold label was frequently found at the outward oriented side of the amorphous plaque structure (Fig. 7, c and d).

**Discussion**

The results reported in this study, together with related observations on desmosomal structures (e.g., 37, 38, 41, 45), show that upon destabilization of intercellular adhesion by depletion of extracellular Ca$^{2+}$ ions, the membrane domains of both kinds of plaque-bearing, cadherin-containing junctions of the “adherens” category, the desmosome and the ZA, behave similarly in principle, as they undergo endocytic vesicle formation in separated vesicle populations. The desmosome-derived, Ca$^{2+}$ depletion-induced vesicles contain transmembrane desmosomal proteins, such as desmoglein (13, 48; see 41), and are associated with desmoplakin-containing plaque material and IFs (8, 13, 37, 38, 41), whereas the ZA-derived vesicles contain E-cadherin in association with plakoglobin (this study), vinculin, and actin-positive plaque material (57). It is to be expected that other proteins of the ZA plaque such as radixin and tennin (for review see 54) and the “282-kD protein” described by Beckerle (I) also remain in association with the ZA-derived endocytotic vesicles.

Compared to the desmosome-derived vesicles, however, the ZA-derived endocytotic vesicles appear to be much less regularly shaped and are less conspicuous amidst the massive mats of the dislocated “outer ring” formed by ZA plaque material and actin filament bundles. Probably, this is the reason why the significance of these vesicles has previously been overlooked (e.g., 57). Mattay and Garrod (41) have described, in the same cell line, a plakoglobin-positive cytoplasmic ring to appear upon EGTA treatment but, as it was also positive for desmocollins, these authors interpreted this ring as exclusively desmosome derived.

Moreover, the labeling of E-cadherin in such ZA-derived vesicles with antibodies to both the extracellular and the intracellular (e.g., by antibodies uvomorulin and pan-cadherin) domains and the demonstration of normal-sized cadherin in immunoblots (data not shown), suggest that most of the cadherin molecules are still intact (for at least 2 h) after Ca$^{2+}$ depletion. On the other hand, our data do not exclude some cadherin degradation during the cell treatment, which is likely to occur in view of the known susceptibility of Ca$^{2+}$-deprived E-cadherin to proteolysis (see 21, 34, 52). Our results obtained with ZA- as well as with desmosome-derived vesicles also do not exclude the possibility that a certain, perhaps large, proportion of E-cadherin and desmoglein molecules escapes the endocytosis process and remains on the outer plasma membrane, apparently in a dispersed distribution over most of the cell surface, due to lateral diffusion (for discussion see also 41). Future experiments will have to show whether the fate and the life time kinetics of the vesicle-trapped and the surface membrane-bound molecules are similar or different, and whether they are reutilized for the reformation of junctions during restoration of the normal Ca$^{2+}$ level. Our experiments indicate that at least for a period of ~40 min after EGTA application the endocytosed ZA-derived vesicles and the vesicles of the “normal” endocytotic pathway represent different and distinguishable compartments. Our results, however, do not allow us to exclude a possible fusion of these vesicle populations in the first minutes of the EGTA treatment.

At present, we do not know the forces that are involved in these rapid endocytic resumption of junctional domains, but it seems reasonable to speculate that the associated cytoplasmic plaque proteins and the specific cytoskeletal filaments play a major role in the stable coherence with the transmembrane glycoproteins and in the maintenance of the differential clustering of E-cadherin vs. desmoglein in the specific type of vesicle. At any rate, our results show that the specific cluster formation and sorting of these two members of the cadherin family of cell adhesion molecules is not depending on homophilic cell-to-cell interaction at the surface, at least as concerns the stability of the clusters once established.
Figure 7. Immunoelectron microscopy of MDBK cells incubated with E-cadherin (uvomorulin; a and b) or pan-cadherin (c and d) antibodies, at 15 min (a and b) or 30 min (c and d) after addition of 4 mM EGTA to the culture medium as detected by gold-labeled (~5 nm diameter) secondary antibodies. Cells were lysed with 0.1% Triton-X-100 in PBS in the presence of 3% formaldehyde. Due to the fixation protocol membrane profiles are not always well preserved. (a) Survey picture showing a dissociated cell with partially detached plaque material (arrowheads) and gold label inside an internalized plaque-associated vesicle (a, arrow; b, at higher magnification). (c) After incubation with pan-cadherin antibodies, reacting with cytoplasmically oriented portions of the molecule, gold label was found at cytoplasmic surfaces of some plaque-associated vesicles (arrows). Inset in c, higher magnification of an area selected from c. (d) Similar situation as shown in c. For better detection, the gold particles were demarcated by small circles. Note preferential orientation of the gold label, which is almost exclusively located on the "outer side" of the plaque. Bars: (a) 1 µm; (b, c, inset in c and d) 0.2 µm.
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