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Dynamics of Tight and Adherens Junctions Under EGTA Treatment

B. Rothen-Rutishauser[1,2], **F.K. Riesen**[1], **A. Braun, M. Günthert, H. Wunderli-Allenspach**
Biopharmacy, Department of Applied BioSciences, ETH Zurich, CH-8057 Zurich, Switzerland

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Abstract. The dynamics of tight junctions (TJs) and adherens junctions (AJs) under EGTA treatment were investigated in Madin Darby canine kidney (MDCK) cells. Detailed information about the behavior of TJ and AJ proteins during the opening and resealing of TJs and AJs is still scarce. By means of the "calcium chelation" method, the distribution and colocalization of junctional proteins were studied with confocal laser scanning microscopy using a deconvolution algorithm for high-resolution images. Colocalization was analyzed for pairs of the following proteins: ZO-1, occludin, claudin-1, E-cadherin and F-actin. Significant differences were found for the analyzed pairs in control cells compared to EGTA-treated cells with respect to the position of the colocalization maxima within the cell monolayers as well as with respect to the amount of colocalized voxels. Under EGTA treatment, colocalization for ZO-1/occludin, ZO-1/claudin-1, claudin-1/occludin, E-cadherin/occludin and E-cadherin/claudin-1 dropped below 35% of the control value. Only for the ZO-1/E-cadherin pair, the amount of colocalized voxels increased and a shift to a more basal position was observed. During the opening of TJs and AJs, ZO-1 colocalized with E-cadherin in the lateral membrane region, whereas in controls, ZO-1 colocalized with occludin and claudin-1 in the junctional complex. The combination of deconvolution with colocalization analysis of confocal data sets offers a powerful tool to investigate the spatial relationship of TJ and AJ proteins during assembly and disassembly of cell-cell contacts.

Key words: MDCK cells — Confocal laser scanning microscopy — Tight junctions — Adherens junctions — Calcium chelation — Colocalization

Introduction

Tight junctions (TJs)[3] form a seal between the apical and the basolateral membrane domain and thus create a barrier to the diffusion of solutes through the paracellular pathway (Cereijido et al., 1998; Madara, 1998). They are complex structures composed of a series of transmembrane and peripheral membrane proteins (reviewed by Mitic & Anderson, 1998). The transmembrane proteins of the TJs such as occludin (Furuse et al., 1993), claudin 1-20 (Furuse et al., 1998) and JAM (Martin-Padura et al., 1998) extend into the paracellular space and thus are candidates for creating the seal. The plaque of membrane-associated TJ proteins, such as ZO-1 (Stevenson et al., 1986), is suspected to be responsible for scaffolding the transmembrane proteins, creating a link to the perijunctional actin cytoskeleton and transducing regulatory signals that control the paracellular barrier (Fanning et al., 1999). Adherens junctions (AJs), another type of intercellular junctions, are located basally to the TJs (Farquhar & Palade, 1963). They are involved in the mechanical linkage of adjacent cells, as well as in the regulation of the development of cell surface polarity (Nelson, 1994). AJs consist of clusters of transmembrane proteins, which belong to the cadherin family. They function as calcium-dependent adhesion molecules and are intracellularly linked to catenins, which in turn promote anchoring to the actin cytoskeleton. In epithelial cells, TJs and AJs are related spatially as well as functionally. It has been suggested that cadherin is the initial organizer of TJ strands. On one hand, evidence comes from the fact that extracellularly added antibodies against cadherin inhibit

[1]These authors contributed equally to this report.
[2]*Present address:* Institute of Anatomy, University of Berne, Switzerland

Correspondence to: H. Wunderli-Allenspach; email: wunderli@pharma.anbi.ethz.ch

[3]Abbreviations used: MEM, Eagle's minimum essential medium; MDCK, Madin Darby canine kidney cell line; TEER, transepithelial electrical resistance; FCS, fetal calf serum; TJ, tight junction; AJ, adherens junction; ZO, zonula occludens; cy, cyanine; CLSM, confocal laser scanning microscopy.

formation of TJs (Vestweber & Kemler, 1985; Gumbiner, Stevenson & Grimaldi, 1988). On the other hand, the TJs and AJs may be physically linked, particularly during formation of TJs, via ZO-1, which binds to the cadherin-associated proteins α-catenin (Itoh et al., 1997) and β-catenin (Rajasekaran et al., 1996). During epithelial cellular polarization, E-cadherin and ZO-1 are simultaneously recruited to the primordial form of spot-like junctions at the tips of cellular processes, which show no concentration of occludin. As cellular polarization proceeds, occludin gradually accumulates at the ZO-1-positive spot-like junctions and E-cadherin is sorted out (Yonemura et al., 1995; Ando-Akatsuka et al., 1999). It has been shown with fibroblasts transfected with either occludin or claudins that the cell-adhesive activity of TJs itself is not particularly strong, which may be the reason why cadherin cell-adhesion activity is required for TJ formation and maintenance (Kubota et al., 1999).

TJs and AJs are highly dynamic structures regarding their morphological aspects and physiological behavior (reviewed by Anderson & Van Itallie, 1995). Detailed information about the opening and resealing of TJs and AJs is still scarce. In the present study we applied the so-called "calcium chelation" method (Cereijido et al., 1978; Collares-Buzato et al., 1994), in which extracellular Ca^{2+} is depleted by addition of the chelator EGTA for a short time before Ca^{2+} is replenished. The chelation of extracellular Ca^{2+} induces disassembly of TJs (Martinez-Palomo et al., 1980; Riesen, Rothen-Rutishaser & Wunderli-Allenspach, 2002) and AJs (Kartenbeck et al., 1982). The behavior of different TJ and AJ proteins as well as of F-actin was investigated during the opening and resealing of TJs and AJs by confocal laser scanning microscopy (CLSM). A colocalization analysis was performed for pairs of ZO-1, occludin, claudin-1, E-cadherin and F-actin at different time points of the opening and resealing in MDCK cells, a well characterized cell line with respect to cytoskeleton, TJ and AJ proteins (Gonzalez-Mariscal, Chávez de Ramirez & Cereijido, 1985; Rothen-Rutishauser et al., 1998a). Data acquisition from fluorescently labeled probes was performed at high resolution. A deconvolution algorithm was applied to reduce noise and blur on one hand and to increase resolution on the other. Colocalization analyses of the protein pairs revealed significant differences in the behavior of the investigated proteins.

Materials and Methods

Cell Cultures

MDCK cells strain II (Rothen-Rutishauser et al., 1998a), were grown in Eagle's minimum essential medium (MEM, ICN, EGT Chemie AG, Tägerig, Switzerland) with Earl's salts supplemented with 10% fetal calf serum (FCS, GibcoBRL, Life Technologies, Basel, Switzerland), 1.7 mM L-glutamine, 0.2% $NaHCO_3$ as well as 100 units penicillin/ml and 100 μg streptomycin/ml (GibcoBRL). Experimental cultures were seeded at a density of 10^5 cells/cm^2 and propagated on Falcon® cell culture inserts with a Cyclopore® membrane (0.4 μm pore size, 4.2 cm^2, #3090, Becton Dickinson Labware, Le Pont De Claix, France) for 11 days, which corresponds to stage III cells (Rothen-Rutishauser et al., 1998a). The transepithelial electrical resistance (TEER) was measured with a Millicell-ERS system (MERS 000 01, Millipore, Bedford MA, USA) at 37°C. In the "calcium chelation" experiments (*see below*), TEER was determined before addition of the EGTA and during the experiment as indicated.

Calcium Chelation

For the "calcium chelation" experiments, cell cultures were treated with EGTA at 37°C as described by Cereijido et al. (1978). In brief, the normal, Ca^{2+}-containing (1.8 mM) MEM was removed and cells were rinsed with Ca^{2+}-free Eagle's balanced salt solution (GibcoBRL). Cells were incubated with MEM without FCS, supplemented with 2.0 mM EGTA (EGTA medium). After 20 min, the EGTA medium was replaced with normal, Ca^{2+}-containing MEM and reconstitution of the cell-cell contacts was observed up to 6 hr.

Antibodies and Fluorescent Reagents

The mouse anti-occludin mAb, rat anti-ZO-1 mAb, rabbit anti-ZO-1 pAb and rabbit anti-claudin-1 pAb were purchased from Zymed Laboratories (Gebr. Maechler, Basel, Switzerland). The rat anti-E-cadherin (uvomorulin) mAb was from Sigma (Buchs, Switzerland). The following secondary antibodies were from Chemicon (Juro, Lucerne, Switzerland): goat anti-rat IgG cy5, goat anti-rat IgG cy3, goat-anti rabbit IgG cy5, goat anti-mouse IgG cy5. Goat anti-mouse IgG cy3 was purchased from Sigma and goat anti-rabbit IgG cy3 from Amersham (Little Chalfont, UK). TRITC-phalloidin was purchased from Molecular Probes (Leiden, Netherlands). For the two triple stainings, the combinations of the following primary and secondary antibodies were used: Samples were either stained with a combination of the primary antibodies rat anti-ZO-1/rabbit anti-claudin-1/mouse anti-occludin and the secondary antibodies goat anti-rat IgG cy5/goat anti-rabbit IgG cy3/goat anti-mouse IgG cy2. Alternatively, the staining was done with a combination of rat anti-E-cadherin/rabbit anti-ZO-1 and was detected by the combination of goat anti-rat IgG cy5/goat anti-rabbit IgG cy2/TRITC-phalloidin. For colocalization studies, double labeling of TJ and AJ proteins as well as F-actin was performed as listed in Table 1.

Immunofluorescent Labeling

Cells were labeled and prepared for CLSM according to Rothen-Rutishauser et al. (1998a). Briefly, cell layers were fixed for 15 min at room temperature in 3% paraformaldehyde in PBS (phosphate buffered saline pH 7.4: 130 mM NaCl, 10 mM Na_2HPO_4/KH_2PO_4). Fixed cells were treated with 0.1 M glycine in PBS for 5 min. They were permeabilized with 0.2% Triton X-100 in PBS for 15 min before they were incubated at 37°C with the primary antibody for 60 min and with the secondary antibody for 90 min. Antibodies were diluted in PBS containing 3% bovine serum albumin as listed in Table 1. Preparations were mounted in 0.1 M Tris-HCl (pH 9.5): glycerol (3:7) containing 50 mg n-propyl-gallate per ml (Sigma).

For claudin-1 stainings, the protocol was slightly modified in order to prevent nonspecific staining: after fixation, cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min and then washed 2 times in PBS followed by one washing step with PBS

Table 1. Combinations of primary and secondary antibodies, fluorescent reagent and their dilutions used for colocalization studies

	Primary antibodies	Secondary antibodies and fluorescent reagent
ZO-1/E-cadherin	Rabbit anti ZO-1 (1:100) Rat anti E-cadherin (1:100)	Goat anti rabbit IgG cy5 (1:50) Goat anti rat IgG cy3 (1:50)
ZO-1/F-actin	Rat anti ZO-1 (1:100)	Goat anti rat IgG cy5(1:50) TRITC-phalloidin (1:10)
ZO-1/occludin	Rabbit anti ZO-1 (1:100) Mouse anti occludin (1:100)	Goat anti rabbit IgG cy5 (1:50) Goat anti mouse IgG cy3 (1:50)
ZO-1/claudin-1	Rat anti ZO-1 (1:100) Rabbit anti claudin-1 (1:25)	Goat anti rat IgG cy5 (1:50) Goat anti rabbit IgG cy3 (1:100)
Claudin-1/occludin	Rabbit anti claudin-1 (1:25) Mouse anti occludin (1:100)	Goat anti rabbit IgG cy3 (1:100) Goat anti mouse IgG cy5 (1:50)
E-cadherin/occludin	Mouse anti occludin (1:100) Rat anti E-cadherin (1:100)	Goat anti mouse IgG cy5 (1:50) Goat anti rat IgG cy3 (1:50)
E-cadherin/claudin-1	Rabbit anti claudin-1 (1:25) Rat anti E-cadherin (1:100)	Goat anti rabbit IgG cy5 (1:50) Goat anti rat IgG cy3 (1:50)
F-actin/occludin	Mouse anti occludin (1:100)	Goat anti mouse IgG cy5 (1:50) TRITC-phalloidin (1:10)
F-actin/claudin-1	Rabbit anti claudin-1 (1:25)	Goat anti rabbit IgG cy5 (1:50) TRITC-phalloidin (l:10)
F-actin/E-cadherin	Rat anti E-cadherin (1:100)	Goat anti rat IgG cy5 (1:50) TRITC-phalloidin (1:10)

Antibodies were diluted in PBS or nonfat dry milk as described in Methods.

containing 5% nonfat dry milk. Samples were incubated at 37°C with the primary antibody for 60 min and with the secondary antibody for 30 min. After washing 3 times with PBS containing 5% nonfat dry milk, cells were mounted as described above. Antibodies were diluted in PBS containing 5% nonfat dry milk as described in Table 1.

Confocal Microscopy

Samples were analyzed with a Zeiss LSM 410 inverted microscope (lasers: HeNe 633 nm, HeNe 543 nm, Ar488/514 nm; Zeiss, Oberkochen, Germany). Optical sections at intervals of 0.15 or 0.3 μm were taken with a 63×/1.4 Plan-Apochromat water objective. Image processing was done on a Silicon Graphics O2 workstation using IMARIS, a 3-D multi-channel image processing software for CLSM data sets (Bitplane, Zurich, Switzerland). The contrast and brightness settings of the microscope were kept constant for each pair of labeled proteins during the course of image acquisition throughout a Ca^{2+} chelation series.

Deconvolution and Colocalization Analysis

Deconvolution and colocalization analysis was done as described in Rothen-Rutishauser et al. (1998b). Briefly, CLSM data were recorded simultaneously for pairs of fluorescence signals at the Nyquist frequency of the microscope, which corresponds in our microscope to a lateral sampling distance of 50 nm and an axial sampling distance of 150 nm (Rothen-Rutishauser et al., 1998b). In order to remove blur, to improve resolution and to reduce noise the "Huygens 2" software was applied using a theoretical point-spread function (Scientific Volume Imaging B. V., Netherlands). Colocalization analysis was carried out with the software "Colocalization" (Bitplane AG). For the statistical analysis, confocal images were recorded as described above. For each pair of proteins, the number of colocalized voxels for each layer was calculated for 5 different membrane areas (area: approx. 10 μm × 4 μm, thickness of one optical section: 0.15 μm).

Results

Dynamics of TJs upon Ca^{2+} Chelation

MDCK cells were grown for 11 days and the "Ca^{2+} chelation" experiment was performed as described in Methods. The addition of EGTA medium resulted in a decrease in TEER (Fig. 1). TEER dropped to 30% of the initial value (~200 Ω cm^2) after 20 min EGTA treatment. Replacement of the EGTA medium with MEM, i.e., restoration of the normal Ca^{2+} concentration, led to the recovery of TEER to 60–70% of the initial value within 60 min. After 3 hr it was back to the control value. According to this TEER profile, studies were performed at four time points: (*A*) control at 0 min, before addition of EGTA medium, (*B*) after 20-min exposure to EGTA medium, (*C*) after 1 hr recovery in normal, Ca^{2+}-containing medium, and (*D*) after 6 hr recovery in normal medium.

The influence of EGTA treatment on MDCK cells was visualized by CLSM. The dynamics of TJs was studied in fixed cells with an antibody against the TJ protein ZO-1 (*see* Methods). Figure 2 shows image restorations in the SFP (simulated fluorescence process) mode of the four time points defined above. Images represent cumulative projections of 40 optical sections of 0.15 μm each. In normal medium (time point *A*), TJs appeared as a continuous network (Fig. 2*a*, *a'*). Under Ca^{2+} chelation, cells began to detach from each other and to round up (time point *B*, Fig. 2*b*, *b'*). At higher magnification, ZO-1-positive connections could be observed between neighboring cells (Fig. 2*b'* arrows). ZO-1 always remained localized at

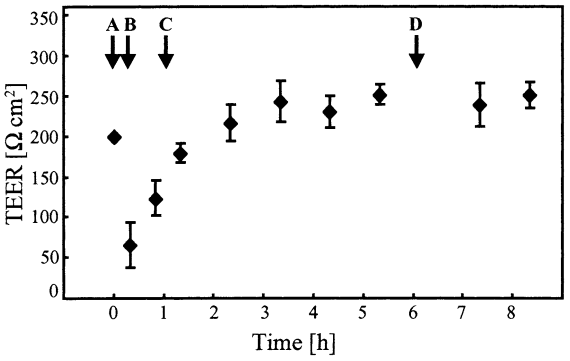

Fig. 1. Effect of calcium chelation on TEER. MDCK cells were grown for 11 d and treated with the "Ca^{2+} chelation" method as described (Methods). TEER values were determined at the times indicated. Four time points were defined for further studies: *A*, control at 0 min before addition of EGTA medium; *B*, after 20 min in EGTA medium; *C*, 1 hr after replacing EGTA medium with normal, Ca^{2+}-containing medium; *D*, 6 hr after replacing EGTA medium with normal, Ca^{2+}-containing medium. Each data point represents the mean ± standard deviation of 5 individual cell culture inserts of one representative experiment.

the cell membranes. Fluorescent strands appeared slightly swollen as compared to the control preparation. After replacement of the EGTA medium with normal, Ca^{2+}-containing medium, contacts between cells were reestablished within 60 min (time point *C*, Fig. 2*c*, *c'*). The slight swelling persisted. A complete network, indistinguishable from the control kept in normal medium, was restored within 6 hr (time point *D*, Fig. 2*d*, *d'*). Inhibition of protein synthesis by addition of cycloheximide to the cell cultures did not influence the outcome of the "Ca^{2+}-chelation" experiment (*data not shown*).

Localization of TJ and AJ Proteins During Opening and Reformation of Cell Junctions

The localization of the TJ and AJ proteins ZO-1, occludin, claudin-1, and E-cadherin, as well as F-actin, was studied in fixed cells at the time points *A–D* (*see* Fig. 1). Preparations were triple-stained for ZO-1/occludin/claudin-1 or ZO-1/E-cadherin/F-actin. ZO-1 served as a reference in both types of preparations. Areas of interest were chosen for each time point and optical sections were taken at the level of the junctional complex for each of the three labeled proteins (Fig. 3). In control cultures, before EGTA treatment, ZO-1 and occludin were exclusively found at the cell-cell contacts, whereas claudin-1 was localized at the cell-cell contacts as well as along the lateral membranes (*data not shown*). E-cadherin showed a bright signal at the cell-cell contacts and in addition a weak signal in the cytoplasm. A similar distribution was found for F-actin except that

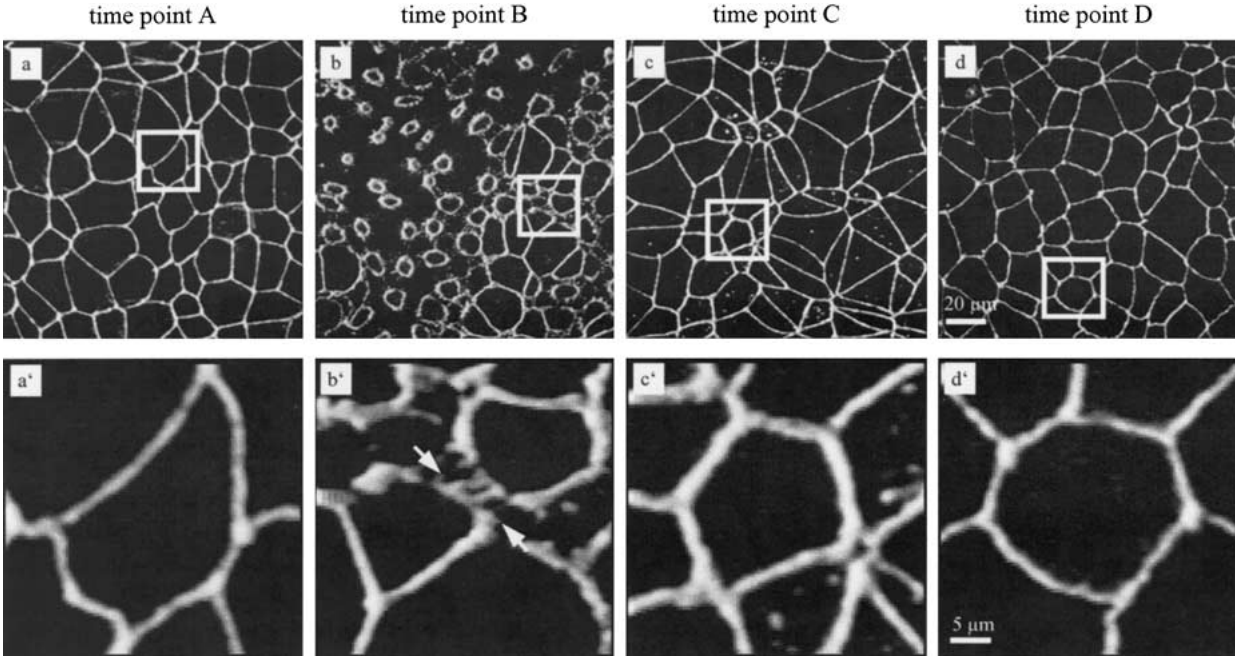

Fig. 2. Effect of calcium chelation on the localization of ZO-1. Cells were cultured and treated with the Ca^{2+}-chelation method as described in Methods. Cells were fixed at time points *A–D* (*see* Fig. 1) and labeled with an anti-ZO-1 antibody (*see* Methods). Micrographs were taken in the CLSM: *a*, *b*, *c*, *d* show 3-*D* reconstructions (SFP mode) to illustrate the ZO-1 network at time points *A–D*; *a'*, *b'*, *c'*, *d'* show a selected region (*see* insets) of *a–d* at higher magnification. The arrows in *b'* point to ZO-1-positive connections between two neighboring cells.

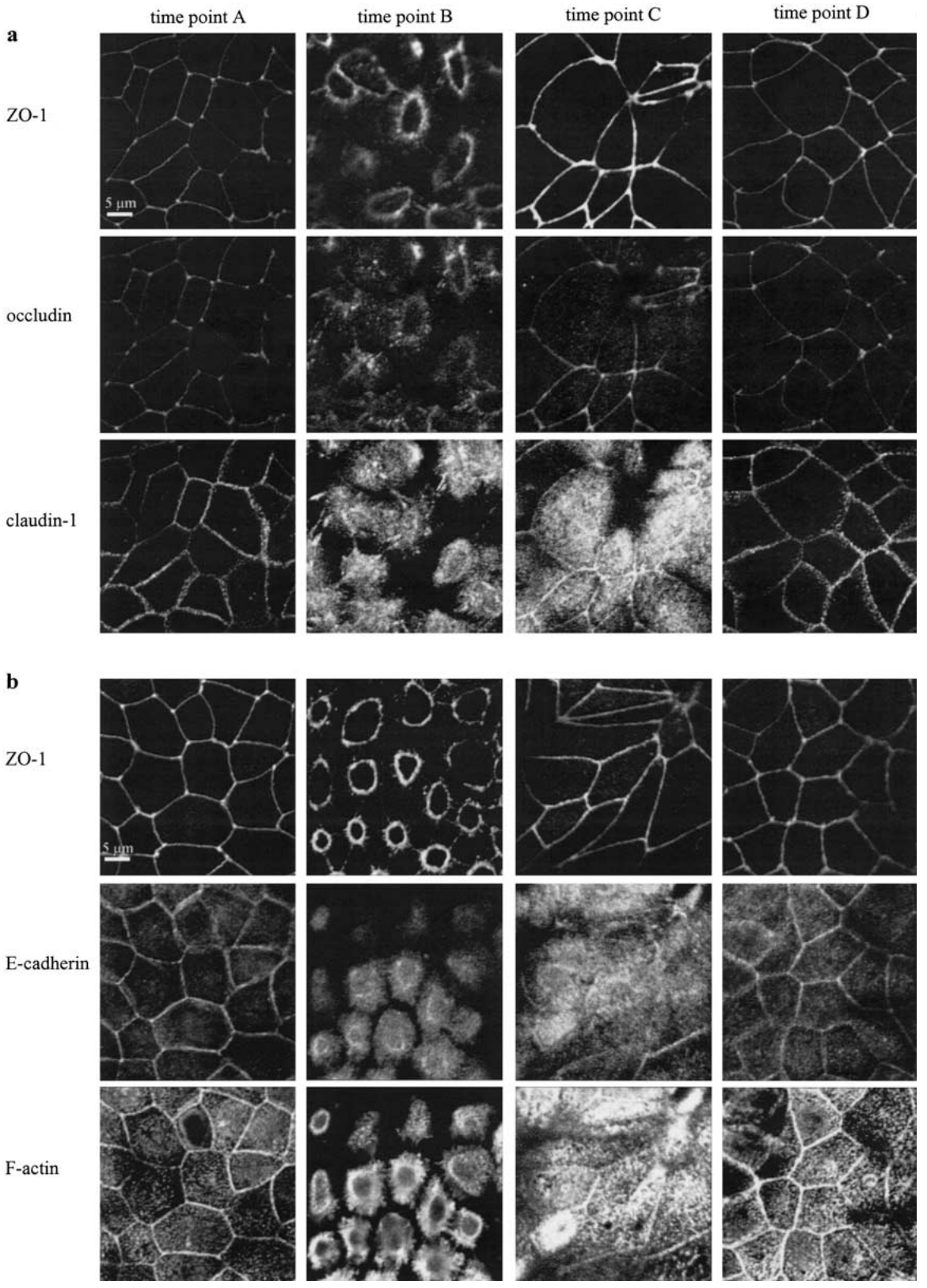

Fig. 3. Effect of EGTA treatment on the localization of TJ/AJ proteins and F-actin. Cells were cultured and treated with the Ca^{2+}-chelation methods. Cells were fixed at the four time points (*A*, *B*, *C*, *D*) defined in Fig. 1 and triple-stained for ZO-l/occludin/claudin-1 (*a*) and ZO-1/E-cadherin/F-actin (*b*), respectively, as described (Methods). Pictures were taken in the CLSM. Single optical sections (x,y) are shown. The contrast and brightness settings of the microscope remained constant during the course of image acquisition throughout a series with equal staining.

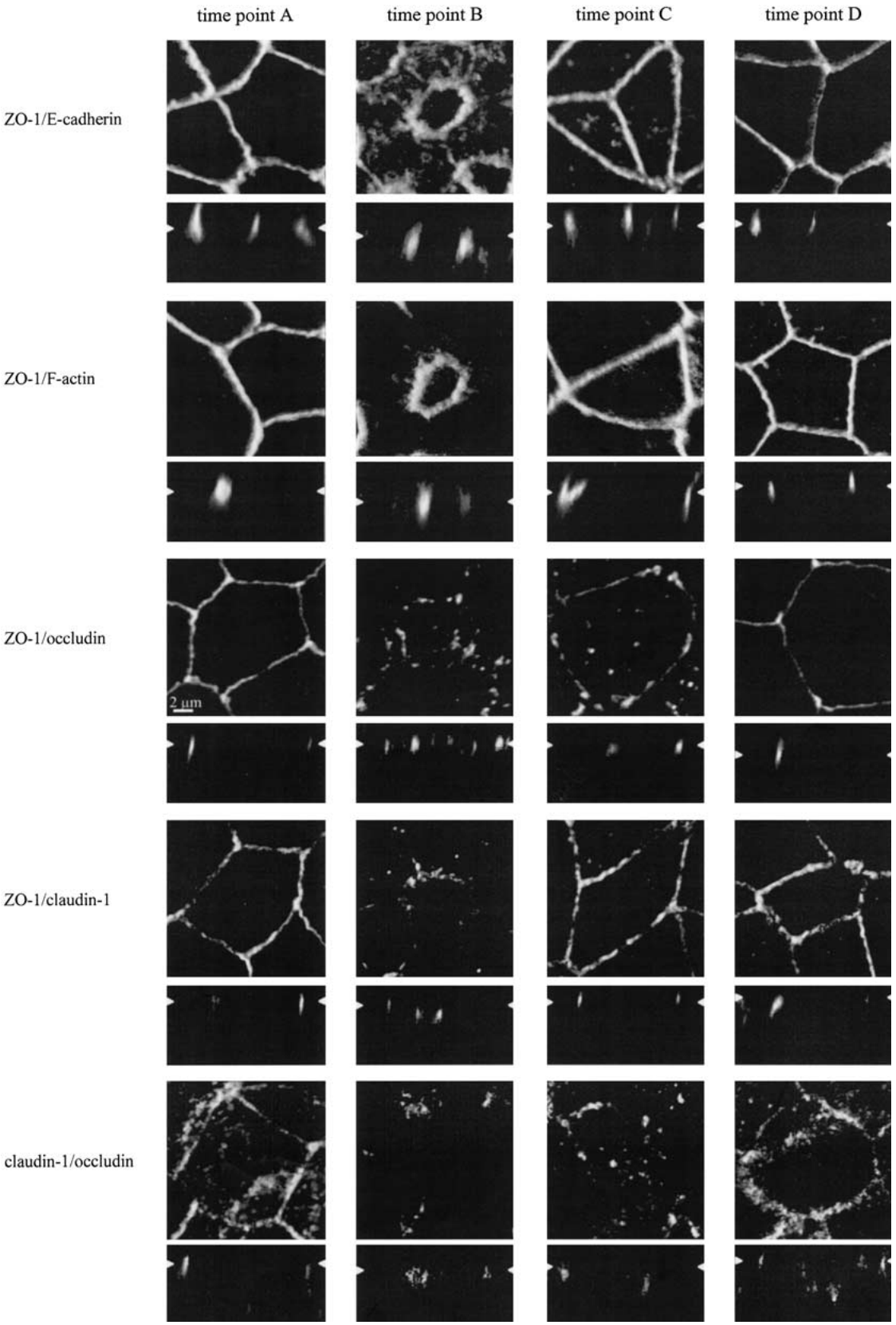

Fig. 4. Colocalization of TJ/AJ proteins and F-actin. Cells were cultured and treated with the Ca^{2+}-chelation method. Cells were fixed at the four time points (*A*, *B*, *C*, *D*) defined in Fig. 1 and double-stained for pairs of TJ and AJ proteins as well as F-actin (*see* Methods). Micrographs were taken in the CLSM at fixed settings and colocalization was analyzed for each protein pair. Colocalization is illustrated as a 3-D reconstruction (upper micrograph) and with the corresponding x,z-projection (lower micrograph) for each time point and each protein pair. Intensities correspond to the cumulative colocalized voxels.

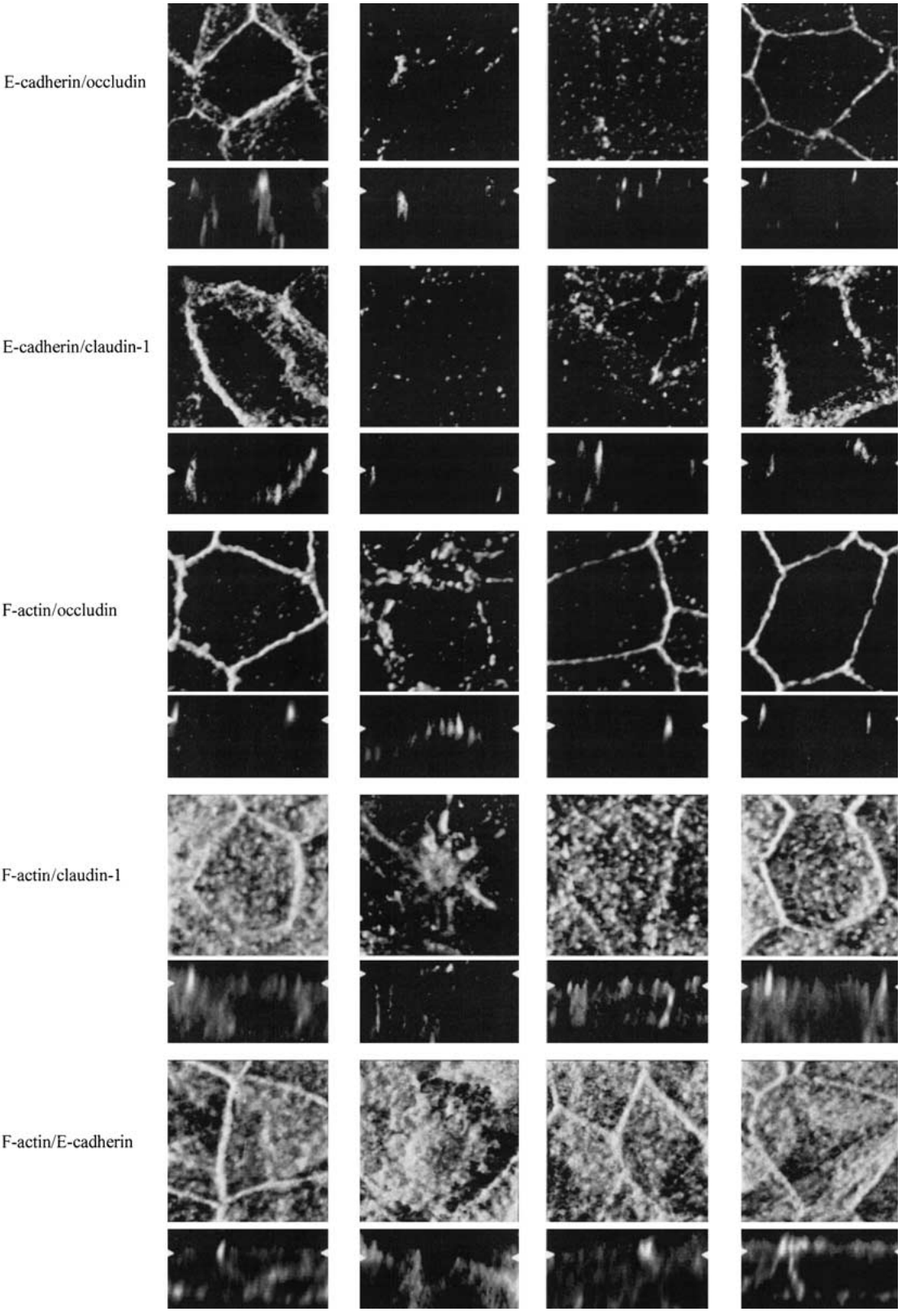

Fig. 4. Continued.

the signal in the cytoplasm was clearly stronger and stress fibers were extending through the basal part of polarized cells (*not shown*).

After 20 min incubation in EGTA medium, when the TJs were opened, occludin showed a similar distribution as ZO-1, however, localization at the membrane was more diffuse than seen with ZO-1. In contrast, claudin-1 and E-cadherin were localized throughout the cells, and F-actin was arranged in a typical peripheral belt. One hour after replacement of the EGTA medium with normal, Ca^{2+}-containing MEM, the reformation of the TJ network was in an advanced state. The complete relocation, indistinguishable from the control, was fast for ZO-1, but took longer for occludin, claudin-1, E-cadherin and F-actin. Only after 6 hr recovery (time point *D*) in normal medium, the network was fully restored and indistinguishable from the control staining pattern.

Colocalization of TJ and AJ Proteins During Opening and Reformation of Cell Junctions

Colocalization studies (*see* Methods) were performed with pairs of proteins in double-labeled samples as indicated in Table 1. The colocalization patterns are visualized in 3D views (SFP mode) from the top of the cells (x,y-orientation) comprising the sum of colocalized voxels, and in a projection (x,z-axis) of the same region of interest (Fig. 4). In control cultures (time point *A*), i.e., before EGTA treatment, two patterns can roughly be distinguished. For ZO-1/E-cadherin, ZO-1/F-actin, ZO-1/occludin, ZO-1/claudin-1, claudin-1/occludin and F-actin/occludin, colocalization was found exclusively at the TJ position, i.e., at cell-cell contacts. The other pairs, namely E-cadherin/occludin, E-cadherin/claudin-1, F-actin/claudin-1 and F-actin/E-cadherin showed colocalization in the TJ position, but also along the lateral membrane, which is clearly seen in the xz-projections. Additionally, F-actin/claudin-1 and F-actin/E-cadherin showed a non-negligible labeling in the cytoplasmic region. With exposure to EGTA medium for 20 min (time point *B*), the colocalization pattern of all protein pairs changed with the most striking differences in the pairs with ZO-1. For ZO-1/E-cadherin and ZO-1/F-actin, colocalization remained confined to the cell membranes, but was extended beyond the cell-cell contacts along the lateral plasma membrane. In contrast, ZO-1 colocalization with the TJ proteins occludin and claudin-1 was reduced to a few spots in the region of cell-cell contacts. The same distribution pattern was found for claudin-1/occludin, E-cadherin/occludin and E-cadherin/claudin-1. For F-actin pairs different patterns were seen. In the case of F-actin/occludin the coherent network along the cell-cell contacts found in the control (time point *A*) was replaced by a beaded pattern of colocalization. For F-actin/claudin-1 and F-actin/E-cadherin, colocalization was reduced in the TJ/AJ area, whereas it remained strong in the cytoplasm. After replacement of EGTA medium with normal, Ca^{2+}-containing medium, colocalization was gradually restored (time point *C*). Colocalization patterns indistinguishable from the respective controls were obtained for all pairs tested within 6 hr recovery in Ca^{2+}-containing medium (time point *D*).

Statistical Analysis of Colocalization of TJ and AJ Proteins During Opening and Reformation of Cell Junctions

To get from the purely descriptive level to a more quantitative statement, the data presented in Fig. 4 were reanalyzed. Five representative membrane regions (area approx. 10 μm × 4 μm, thickness of optical section: 0.15 μm) comprising single cell-cell contacts, i.e., membrane areas, were selected. The number of colocalized voxels of these regions was calculated for each protein pair in each optical section along the z-axis, i.e., through the cell layer (~9 μm). Colocalization profiles of all protein pairs are plotted in Fig. 5. The focus is on time point *A* (control, before addition of EGTA medium) and time point *B* (after 20 min EGTA medium). The full recovery of TJs, documented in Fig. 4, was confirmed with this analysis, but time point *C* and *D* have been omitted for clarity. Despite the relatively large standard deviations, a very clear pattern emerged. Before Ca^{2+} chelation (time point *A*) all tested protein pairs fell roughly into two classes according to their colocalization profile. The four ZO-1 pairs as well as F-actin/occludin exhibited one prominent colocalization peak in the junctional area, i.e., within the layers 1 to 25, close to the apical level. For occludin/claudin-1, E-cadherin/occludin, F-actin/claudin-1 and F-actin/E-cadherin, colocalization was not limited to the junctional area, but extended along the z-axis. A first peak was present in the TJ area in agreement with the other protein pairs. However, a high level of colocalization persisted down to levels 40 to 50. For all protein pairs tested, the colocalization pattern changed significantly under Ca^{2+} depletion. The colocalization peak for ZO-1/E-cadherin and ZO-1/F-actin shifted to a position between layers 30 to 40, while the maximum level of colocalized voxels doubled. For ZO-1/occludin and ZO-l/claudin-1, in contrast, the colocalization peak was reduced to about 15% of the control peak with a shift from around layer 15 to around layer 25. With claudin-1/occludin a similar pattern was found as with ZO-1/occludin and ZO-1/claudin-1. The peak at position 15 was shifted to a position around 25, however, the reduction was not as prominent as with ZO-1/occludin and ZO-1/claudin-1. The second peak (around position 43) encountered in the control preparation of the claudin-1/occludin pair disappeared under EGTA treatment. In

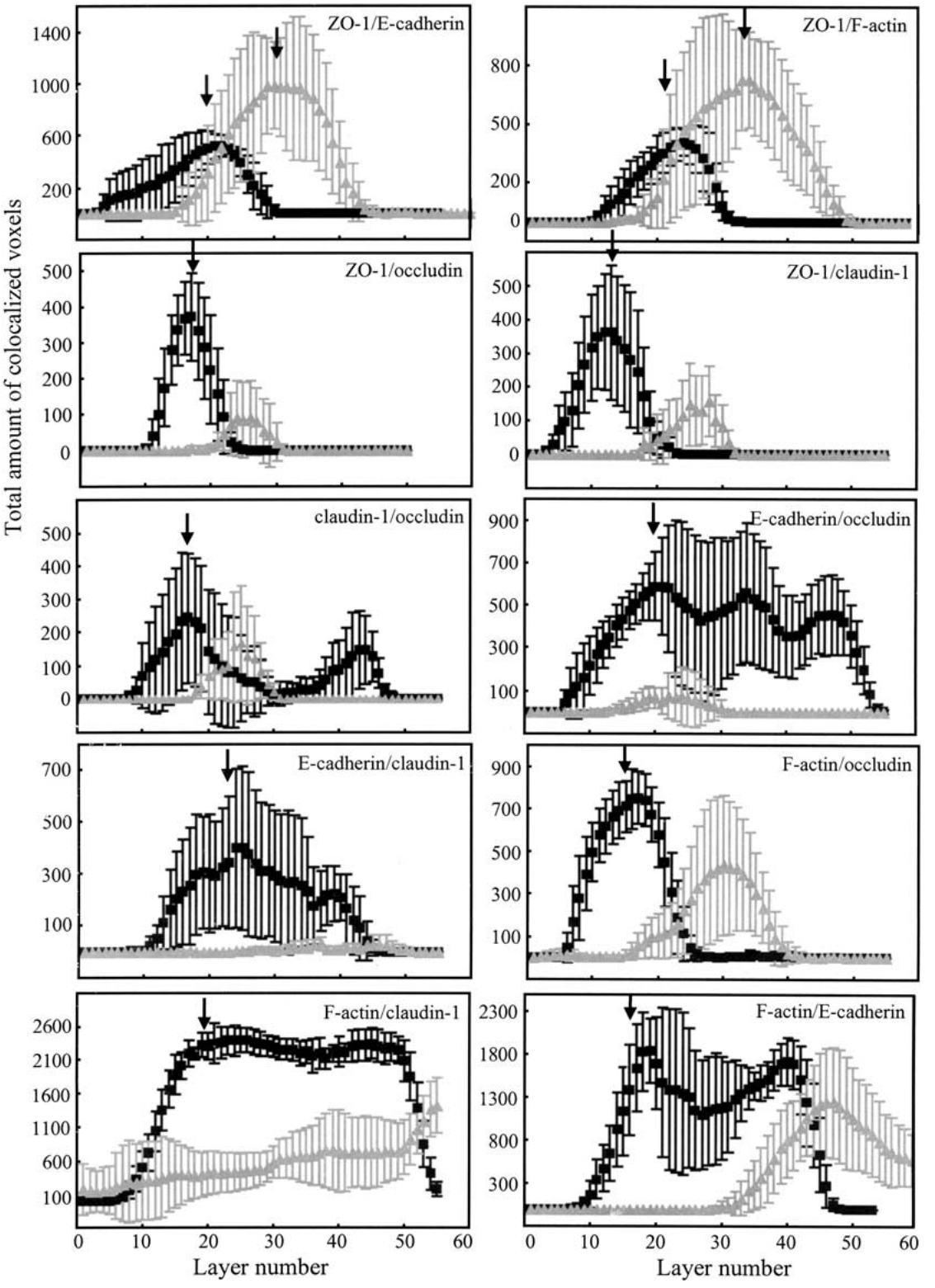

Fig. 5. Dynamics of TJs and AJs during EGTA treatment. From the micrographs shown in Fig. 4, five representative membrane regions (area approx. 10 μm × 4 μm) were selected and the number of colocalized voxels was calculated for each optical layer. The first layer (# 1) corresponds to the optical section at the apical position and the last layer (# 60), to the one at the basal position of the cells. Colocalization plots: time point *A* (black, control), time point *B* (gray, 20 min EGTA treatment). Each data point represents the mean ± standard deviation of the number of colocalized voxels of 5 representative membrane regions. Arrows point to regions of cell-cell contacts.

the case of E-cadherin/occludin and E-cadherin/claudin-1 no significant colocalization remained after EGTA treatment. For F-actin/occludin, a shift of the colocalization peak was noted from layer 15 to layer 30 and the level of colocalization was reduced to about 50%. With F-actin/claudin-1, significant colocalization was found in a broad peak between layers 20 to 50 in the controls. It was reduced to below 20% of the control level. Finally, with F-actin/E-cadherin, the first peak of colocalization (around layer 20) disappeared completely, whereas the second peak (around position 40) remained, though slightly shifted.

For better comparison of the changes in colocalization between the protein pairs, the total amount of colocalized voxels (xyz-space; about 6 μm^3) was analyzed for selected pairs (Fig. 6). Time point *A* of each pair was used as internal control and the respective numbers of colocalized voxels at time point *B* were normalized to the control values. This analysis shows an increase (between 220 and 280%) in the number of colocalized voxels for ZO-1/E-cadherin and ZO-1/F-actin. In contrast, for ZO-1/occludin and ZO-1/claudin-1, a significant decrease of colocalized voxels to less than 30% of the control was observed. The same trend was found with claudin-1/occludin, although the difference was not statistically significant in this case. In the case of E-cadherin/occludin and E-cadherin/claudin-1, colocalization disappeared completely under EGTA treatment. For the remaining F-actin/occludin, F-actin/claudin-1 and F-actin/E-cadherin pairs there was a tendency for a reduction of colocalized voxels. In the case of F-actin/occludin, it was not statistically different from the control value.

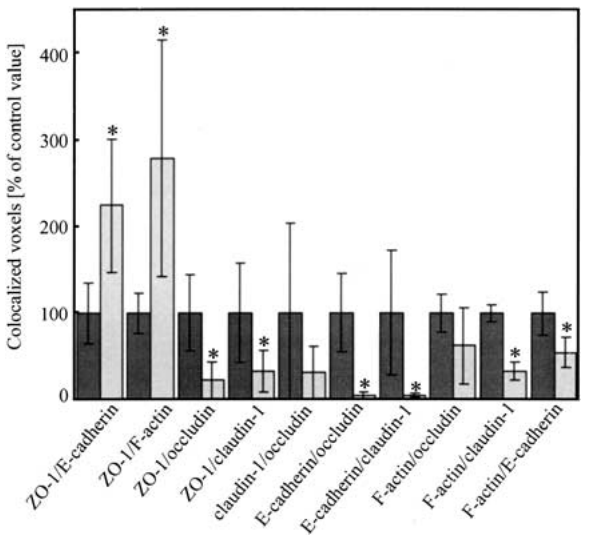

Fig. 6. Statistical analysis of colocalization during EGTA treatment. Cells were treated with the Ca^{2+}-chelation method and prepared for colocalization analysis in the CLSM as described in Fig. 3. For all pairs, representative membrane regions (app. 10 $\mu m \times 4$ μm) were analyzed (*see* Fig. 5) and the total number of colocalized voxels calculated (x,y,z-space): time point *A* (black, control) and time point *B* (gray, 20 min EGTA treatment). For each protein pair the numbers were normalized to the number of colocalized voxels in the control (time point *A*) and expressed as percentage. Means ± standard deviations of the number of colocalized voxels were calculated for each protein pair from 5 representative membrane regions. Colocalization values of pairs with 95% significance compared to the respective control, determined by a two-sample test, were labeled with an asterisk.

Discussion

The colocalization pattern of all TJ and AJ protein pairs, established by CLSM and subsequent image analysis, changed significantly during the opening of the junctions. Differences were most striking between the four ZO-1 pairs: ZO-1/E-cadherin, ZO-1/F-actin, ZO-1/occludin and ZO-l/claudin-1. In control cell cultures, i.e., before the addition of EGTA, colocalization for all ZO-1 pairs was found exclusively near the apical domain at the cell-cell contacts within the TJ belt. Our data confirm the colocalization between ZO-1 and F-actin found by Fanning et al. (1998) and Wittchen et al. (1999) using a combination of in vitro and in vivo binding assays. Data are also in agreement with previous colocalization and in vitro binding studies that showed binding of ZO-1 to claudin-1 (Itoh et al., 1999) and occludin (Furuse et al., 1994). It is interesting to note that colocalization of ZO-1/E-cadherin is very similar to that of ZO-1/F-actin although there is no evidence for a direct association of ZO-1 with E-cadherin. During EGTA treatment, i.e., opening of the junctions, colocalization of ZO-l/E-cadherin and ZO-1/F-actin remained confined to the cell membrane, but extended along the lateral membrane beyond the original areas of cell-cell contacts. These findings could be substantiated by statistical analysis. The amount of colocalized voxels increased significantly (2–3-fold) and a shift of the colocalization maxima to a more basal position was observed. For ZO-1/occludin as well as ZO-1/claudin-1, in contrast, colocalization was reduced to a few spots in the region of the cell-cell contacts (~15% of the control value). This indicates that upon opening of TJs and AJs the colocalization pattern of ZO-1 with other junctional proteins changes completely. The original colocalization of ZO-1 with occludin and claudin-1 disappears, an increased colocalization with E-cadherin is established. No information is available from other studies for the possible colocalization of these proteins during the opening of established TJs and AJs. Previous colocalization studies had been concentrating on the establishment of new cell-cell contacts of cultured epithelial cells for ZO-1, occludin and E-cadherin (Yonemura et al., 1995; Rajasekaran et al., 1996; Ando-Akatsuka et al., 1999). The authors could demonstrate that ZO-1 associates initially with AJ components prior to the final localization at TJs, and evidence was presented that in ZO-1-positive

junctions the amount of E-cadherin decreased, while occludin increased as epithelial polarization proceeded. These results are consistent with our findings and can be interpreted that ZO-1 colocalizes with E-cadherin as long as no functional TJs exist. They confirm the importance of ZO-1 in the (re)-formation of TJs during epithelial cellular polarization.

For the integral membrane proteins occludin and claudin-1, colocalization in control cells was restricted to the cell-cell contacts. These observations confirm the close positional relationship between occludin and claudin-1 as already shown by Furuse et al. (1998) by means of a colocalization approach with cross-sectional views. During the opening of TJs with EGTA, colocalization disappeared. The colocalization data for ZO-1, occludin and claudin-1 indicate a complete rearrangement of the TJ-related proteins upon opening of the TJs.

We have also shown that in control cells occludin and claudin, respectively, colocalized with E-cadherin near the apical domain at the cell-cell contacts. During EGTA treatment this colocalization disappeared. No published data are available for these pairs of proteins.

Before EGTA treatment, occludin, claudin-1 and E-cadherin, respectively, colocalized with F-actin, similar to observations with ZO-1/F-actin. In contrast to ZO-1/F-actin, there is no indication for a direct binding of occludin, claudin-1 and E-cadherin to the actin filament, whereas it is known that F-actin is associated through ZO-1 with occludin and claudin-1 (Fanning et al., 1998; Itoh et al., 1999) and through α-catenins with E-cadherin (Rimm et al., 1995). Colocalization was significantly reduced during EGTA treatment for F-actin/occludin, claudin-1/F-actin and F-actin/E-cadherin. Taken all together, we postulate that during EGTA treatment ZO-1 still binds to F-actin, whereas the binding of ZO-1 to occludin and claudin-1 is lost and therefore also the spatial relationship of occludin and claudin-1 to actin filaments.

In summary, we could demonstrate that in established cell-cell contacts, ZO-1 colocalized with occludin and claudin-1 in the junctional complex, whereas upon disruption of the TJs, enhanced colocalization of ZO-1 with E-cadherin in the lateral membrane region appeared. Furthermore, we showed that colocalization between occludin, claudin-1, E-cadherin and F-actin disappeared during the opening of the junctions.

The origin of the different binding behavior of ZO-1 to occludin, claudin-1 and E-cadherin in established cell-cell contacts and during the opening of the junctions is not clarified yet. The peculiar distribution of ZO-1 can be partly explained by the affinity of its N-terminal half for the C-terminal sequence of occludin (Furuse et al., 1994) and for the C-terminal sequence of α-catenin, which binds directly to E-cadherin (Itoh et al., 1997). However, it still remains unclear why, during the opening of the junctions, ZO-1 is recruited to AJ proteins, whereas in polarized epithelial cells, ZO-1 is localized at TJs, although α-catenin is still concentrated at the AJs. Further analysis of the molecular basis should clarify this behavior. It is likely that the different binding behavior of ZO-1 to E-cadherin is due to changes in the conformation of the E-cadherin/catenin complex. It has been suggested that Ca^{2+} does not only work as a simple bridge between junctions but also affects E-cadherin to undergo Ca^{2+}-dependent conformational changes (Vestweber & Kemler, 1985; Pokutta et al., 1994; Nagar et al., 1996). Furthermore, Citi (1992) showed that protein kinase inhibitors prevent opening of cell junction. Even though it has been shown that ZO-1 phosphorylation does not change when Ca^{2+} is removed for a short time (Howarth et al., 1994), there are still many potential points of attack for kinases and phosphatases in the junctional complex.

Colocalization analysis provides information about the spatial arrangement of two proteins. Since the resolution is limited to about 50–100 nm (Rothen-Rutishauser et al., 1998b), we are restricted to show close proximity but not a direct association of two different proteins. Best evidence for a direct association between different junctional proteins would be the demonstration of binding interactions with biochemical techniques, such as immunoprecipitation or co-sedimentation. Few results were obtained with these methods, since the junctional transmembrane proteins are polymerized in detergent resistant strands (Itoh et al., 1999) and protein-protein interaction may not be strong enough to be demonstrated by means of immunoprecipitation. Another promising method for quantitative colocalization studies would be FRET (fluorescence resonance energy transfer) analysis using different GFP fusion proteins with TJ and AJ proteins (Pollok and Heim, 1999). It would have to be demonstrated, however, that the different GFP entities would not interfere with the localization and mutual interactions of proteins.

The combination of CLSM and deconvolution with subsequent colocalization analysis and statistical evaluation represents a powerful tool to explore the relative spatial arrangement of protein pairs and provides the means to follow shifts of colocalization maxima along the x,z- and y,z-axis. Even though we are limited to show close proximity instead of direct association of two different proteins, this method helps, on a comparative basis, to get a better understanding of the dynamics of the junctional complexes.

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