Changes in Membrane–Microfilament Interaction in Intercellular Adherens Junctions upon Removal of Extracellular Ca^{2+} Ions

Tova Volberg,* Benjamin Geiger,* Juergen Kartenbeck,‡ and Werner W. Franke‡

*Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel, and ‡Division of Membrane Biology and Biochemistry, Institute for Cell and Tumor Biology, German Cancer Research Center, 6900 Heidelberg, Federal Republic of Germany

Abstract. EGTA-induced depletion of Ca^{2+} ions from the culture medium of Madin-Darby bovine kidney epithelial cells results in rapid splitting of adherens-type junctions and the detachment of the vinculin- and actin-containing filament bundle from the cytoplasmic faces of the plasma membrane of the *zonula adhaerens*. This process was monitored by phase-contrast microscopy, combined with electron microscopy and immunofluorescent localization of the two proteins. It is shown that shortly after extracellular free Ca^{2+} concentration is lowered to the micromolar range, the actin-containing, junction-associated belt of microfilaments, together with the vinculin-rich junctional plaque material, is irreversibly detached as one structural unit from the plasma membrane, contracts, and is displaced towards the perinuclear cytoplasm where it gradually disintegrates. Other actin- and vinculin-containing structures present in the same cells, notably the focal contacts at the substratum, are not similarly affected by the Ca^{2+} depletion and retain both the adhesion to the external surface and the association with the plaque and microfilament components. Electron microscopic examination has shown that the membrane domain of the *zonulae adhaerentes*, unlike that of desmosomes, is not endocytosed after Ca^{2+} removal and that the displaced actin- and vinculin-containing plaque and filament belt are not associated with a particular membrane. It is further shown that upon restoration of normal Ca^{2+} levels in the culture medium, new intercellular contacts are established gradually by accretion of both vinculin and actin into new belt-like plaque- and microfilament-containing structures.

ADHERENS-TYPE junctions are a family of cell contacts which are associated at their cytoplasmic surfaces with actin-containing microfilaments (10, 20, 40). Attempts to define the nature of actin binding to the membrane have indicated that the junction-associated microfilaments are assembled into a bundle and are connected with the plasma membrane through a vinculin-rich "plaque" structure (4, 14, 16–20). Cell contacts of this category can differ in their general appearance, size, and cellular topology and include the continuous, belt-like junctions (*zonulae adhaerentes*) of polarized epithelia, the patchy, streak-like junctions (*fasciae adhaerentes*) of cardiac myocytes, and small punctate attachments (*puncta adhaerentia*) detached in intact tissues and in culture cells. Adherens contacts are formed also with extracellular materials both in intact tissues and in cell cultures (11, 26, 27, 37, 40); the best characterized example for such attachments are the focal contacts of cultured cells (1, 8, 25, 29, 30).

Studies of the different molecular constituents of adherens junctions, their interrelationships and interactions have suggested that these sites contain multimolecular transmembrane complexes. On the basis of immunoelectron microscopic data (18, 19), immunofluorescent labeling (i.e. 4, 14, 38, 42) and specific studies of the removal of actin from isolated cell contacts (2, 21), we have proposed that adherens-type junctions consist of at least three major subdomains: these are one or several "contact-specific membrane receptors," an intracellular membrane-bound junctional plaque that contains vinculin and, in some cases, talin (5, 6) and a cytoskeletal element composed of actin filaments and several actin-associated proteins (i.e., α-actinin [21, 34]). We have further proposed that formation of local contacts may trigger a chain of interactions between elements of neighboring subdomains, leading to their assembly into an intact transmembrane junctional complex (for discussions see references 16, 22, and 24).

One of the critical steps in junction biogenesis is the initial "transmembrane event," i.e., the first local change that occurs at the cytoplasmic surfaces of the junctional membrane, the assembly of the junctional plaque. To study this process, we have exposed cultures of polar epithelial cells to Ca^{2+}-depleted medium and examined the differential disintegration of the various junctional elements. Such treatment has been previously shown to specifically disrupt the integrity of several types of intercellular junctions (notably desmosomes) (31,

32), and to abolish transepithelial resistance (7, 12, 36, 39).

In a previous study, we have shown that, shortly after chelation of extracellular Ca^{2+} from cultured Madin-Darby bovine kidney (MDBK)[1] epithelial cells, desmosomal junctions were split and the half-desmosomes thus formed subsequently endocytosed (32). Concomitantly, the *zonulae adhaerentes* disintegrated and vinculin was apparently translocated towards the cell center. However, the exact effects of extracellular Ca^{2+} depletion on the dynamics of vinculin–membrane and vinculin–actin interactions have not been examined in detail.

In this report, we show that within a few minutes after incubation of MDBK cells in media containing only micromolar concentrations of Ca^{2+}, a filamentous belt containing both F-actin and vinculin dissociates as a whole entity from the junctional membrane and appears to move towards the perinuclear area where it subsequently disintegrates. We also show that once the plaque and the bound actin belt have dissociated from the membrane, they do not reattach to the membrane even if normal extracellular Ca^{2+} is restored. This supports the view that re-establishment of new intercellular contacts is essential for the re-assembly of the plaque and cytoskeletal subdomains and the establishment of nascent junctions.

Materials and Methods

Cell Culture

MDBK cells (ATCC, CCL 22) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and maintained in a humidified incubator under an atmosphere of 7% CO_2 and 93% air (41). The calcium ion concentration in the Dulbecco's modified Eagle's medium was 1.4 mM. Treatment with EGTA (Sigma Chemical Co., St. Louis, MO) was initiated by the addition of aliquots from buffered stock solution.

Immunofluorescent Labeling and Microscopy

Cells cultured on 12-mm round coverslips were fixed and permeabilized with formaldehyde and Triton X-100 as described (20, 32). Actin was visualized in the cells by staining with rhodamine-labeled phalloidine, kindly supplied by Dr. H. Faulstich (Max-Planck Institute for Medical Research, Heidelberg, Federal Republic of Germany). Vinculin was localized using a monoclonal mouse antibody (VIN 11.5) and fluorescein- or rhodamine-conjugated goat anti-mouse Fab'_2 (14, 20). Use of fluorescein-labeled antibodies in conjunction with rhodamine–phalloidine enabled co-localization of both vinculin and actin within the same cells. Stained cells were mounted in Elvanol and examined in a Zeiss Photomicroscope III equipped with filter sets for selective fluorescein–rhodamine observation as well as for phase-contrast microscopy.

Electron Microscopy

Cells cultured on 35-mm tissue culture dishes (type 3001; Falcon Labware, Oxnard, CA) were fixed in situ with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The cells were rinsed, postfixed in 1% OsO_4, dehydrated in ethanol, and embedded in a thin layer of Epon (Polybed 812; Polysciences, Inc., Warrington, PA). After 48 h at 60°C, the Epon with the embedded cell monolayer was removed from the dish, re-embedded at a desired orientation (for cross- or parallel-sectioning), cut with a diamond knife (Diatome, Switzerland), and examined in a Philips 410 electron microscope at 80 or 100 kV.

Results

Adherens Junctions in Cultured MDBK Cells

Fluorescent microscopic localization of actin in cultured MDBK cells revealed two major, spatially distinguishable patterns of organization as previously described (20): at the subapical focal plane actin appeared as a peripheral belt running along the cell borders (Fig. 1 *A*), whereas at the ventral aspects of the same cells many stress fibers of variable lengths could be seen (Fig. 1 *B*). Examination of the same group of cells by interference reflection microscopy indicated that the stress fibers invariably terminated in interference reflection microscopy–dark plaques corresponding to focal contacts (compare Fig. 1 *B* to 1 *C*; see also references 1, 8, 25, 29, 30).

Transmission electron microscopy of cultured MDBK cells revealed a thick (0.5–0.8 μm) electron-dense bundle of microfilaments running along the cytoplasmic faces of the subapical cell membrane. This belt bundle was often flanked by intermediate-sized filaments, which in some regions penetrated into it (Fig. 1, *D* and *E*; see also Fig. 4 *B*). Occasionally, in sections including extended areas of the subapical portion of the lateral membrane (Fig. 1 *E*), periodic densities could be seen along the junction-attached microfilament belt bundle with an average center-to-center spacing of ~0.5 μm. At the ventral aspects of the cell, numerous focal contacts were detected with microfilament bundles attached at their cytoplasmic aspects (Fig. 1 *F*).

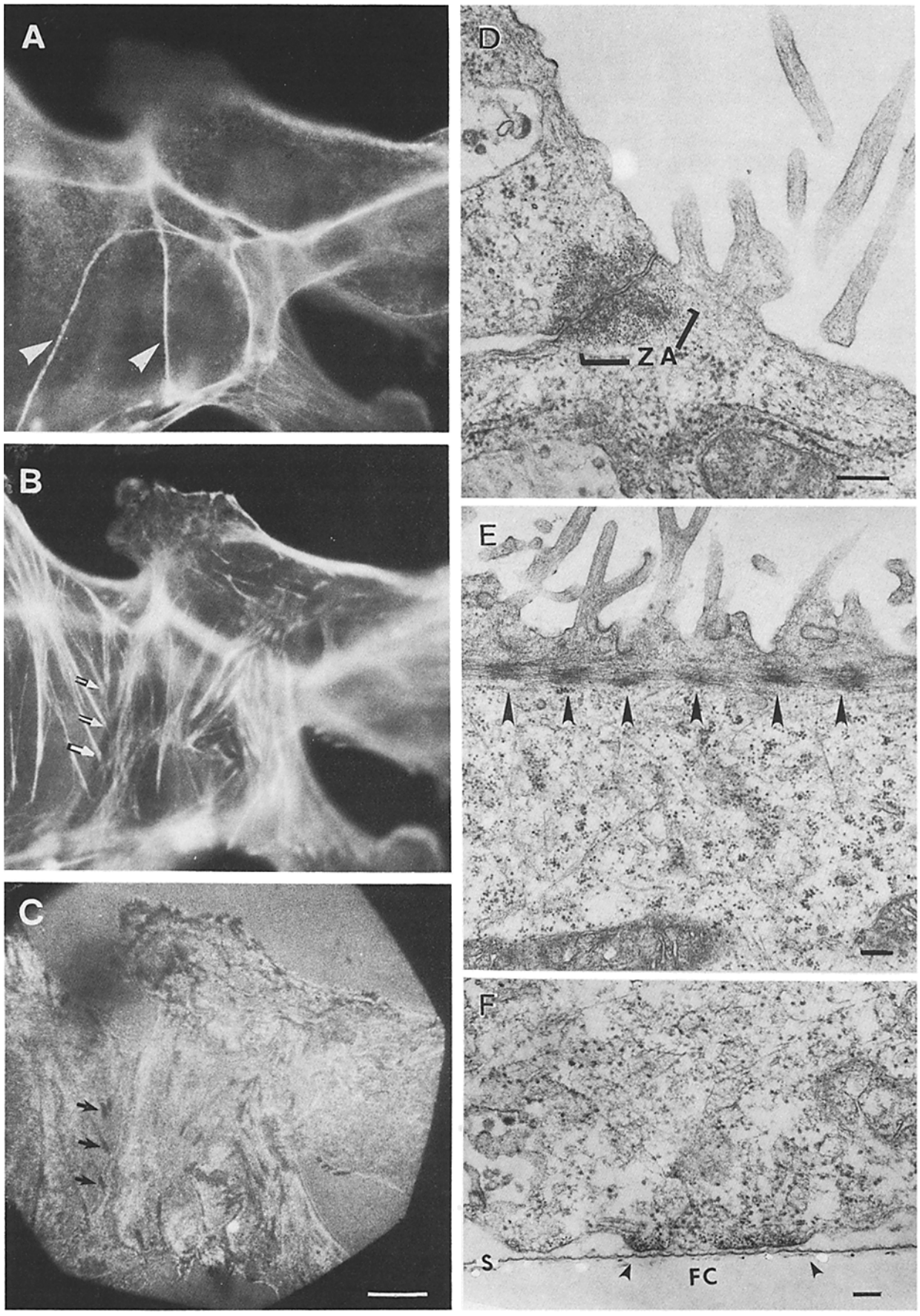
Effect of Ca^{2+} Depletion on Intercellular Junctions

Shortly after addition of 2–4 mM EGTA to the culture medium of MDBK cells, disruption of cell–cell contacts was evident by phase-contrast microscopy (Fig. 2). Examination of the EGTA-concentration dependence indicated that dissociation of intercellular junctions occurred when free Ca^{2+} concentrations was dropped to 4×10^{-6} M or lower. Maximal effect (with respect to the apparent rate of cell separation) was observed at 1.8×10^{-6} M free Ca^{2+} or lower. The dissociation of cell contacts is demonstrated here in a sequence of phase-contrast micrographs taken at different times after Ca^{2+} depletion (Fig. 2). As early as 2–4 min after the addition of EGTA (2 mM), detachment of cells was first evident and cell separation and contraction proceeded for ~10 min until essentially all intercellular junctions, originally seen as phase-dark lines, disappeared. Subsequently, progressive cell rounding was noticed, often leading (after few hours) to partial or complete detachment from the glass or plastic substrates. In the presence of 4 mM EGTA (rather than 2 mM) breakdown of junctions was even faster and apparent already 1 min after initiation of treatment.

Labeling of EGTA-treated cells with rhodamine–phalloidin revealed marked changes in the organization of the junctional belt of actin microfilaments. Initially, the junction-associated actin bundles of two neighboring cells became separated in several regions (Fig. 3 *A*) and, as the process progressed, the entire F-actin filament belt, or at least large parts of it, retracted towards the cell center and apparently dissociated from the plasma membrane (Fig. 3 *B*; see also below). Electron microscopic examination of MDBK cells that were briefly (up to 10 min) treated with EGTA indicated that remarkable splitting of the adherens junctions occurred before dissociation of the actin filament belt was apparent (Fig. 3, *C* and *D*).

A direct demonstration that the actin filament belt in cells treated with EGTA for ~10 min or more indeed detached from the plasma membrane was provided by transmission electron microscopy. In untreated cells, bundles of microfilaments were detected along the subapical junctional mem-

1. *Abbreviation used in this paper:* MDBK, Madin-Darby bovine kidney.


A
B
C
D
ZA
E
F
S
FC

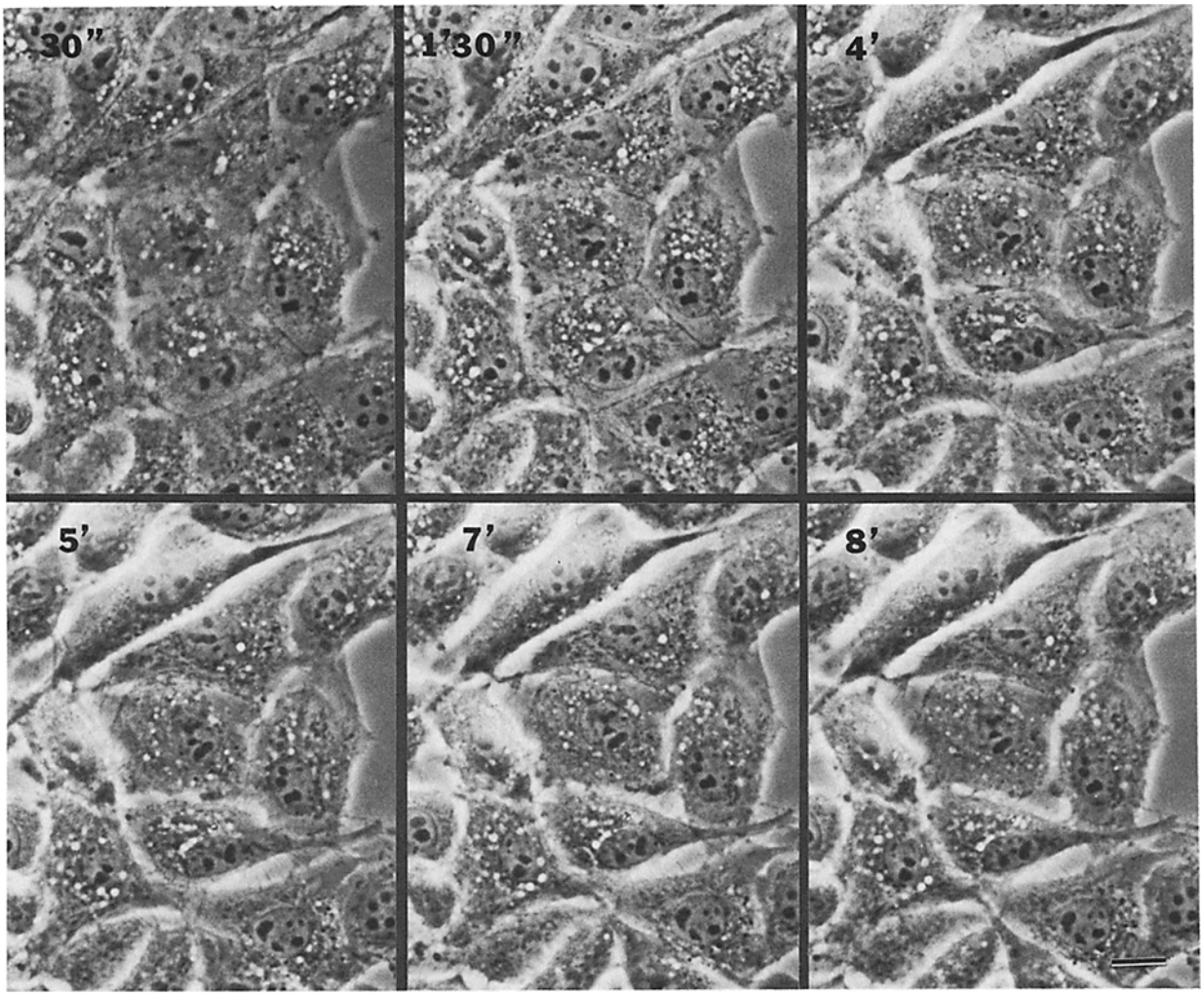


Figure 2. Phase-contrast photomicrographs taken at different intervals after addition of 2 mM EGTA. Notice a progressive breakdown of intercellular junctions which becomes clearly apparent 4 min after addition of EGTA. Bar, 10 μm.

brane, closely associated with its cytoplasmic surfaces (Fig. 4, *A* and *B*, and Fig. 1 to the left). In EGTA-treated cells, on the other hand, the microfilament belt was clearly located deeper in the cytoplasm, not attached to the plasma membrane nor to any other recognizable membrane-containing structure (Fig. 4 *C*). This observation was corroborated by the examination of serial sections of the same regions (not shown). It is noteworthy that the detached microfilament belt, or segments of it, were usually convoluted (Fig. 4, *D* and *E*), in contrast to the microfilaments bundle of the untreated cells which was largely straight (Fig. 4, *A* and *B*). The periodic center-to-center spacing of electron densities along the detached bundle was reduced to ~0.3 μm, suggesting that contraction of the belt has indeed taken place. As pointed out above, the actin detachment process lags behind the disruption of cell contacts (e.g., Fig. 3, *C* and *D*). As shown above, remarkable dissociation of cell–cell contacts was evident within few minutes after Ca^{2+} depletion whereas significant dissociation of the actin bundle from the plasma membrane required treatment for 10 min or more.

Double fluorescent labeling for actin and vinculin of untreated or EGTA-treated MDBK cells indicated that the two proteins were closely associated with each other before and after the EGTA-induced dissociation of the microfilament belt structure from the junction (Fig. 5). As previously shown, actin and vinculin of untreated MDBK cells are present at

Figure 1. Microfilament organization in cultured MDBK cells. (*A–C*) The same group of cells labeled for actin with rhodamine-phalloidine (*A* and *B*, photographed at the apical and ventral focal planes, respectively) or examined by interference reflection microscopy (*C*). The arrowheads in *A* point to the actin-rich microfilament belt while the matching arrows in *B* and *C*, point to sites of cell-substrate focal contact. (*D–F*) Transmission electron micrographs showing the filament bundles at the *zonula adhaerens* (*ZA*) in cross section (*D*) or longitudinal section (*E*). Notice the periodic electron densities along the belt (arrowheads) with an average spacing of ~0.5 μm. The focal contact (*FC*) in *F* is associated with many microfilaments (appear here mostly in cross section) and commonly flanked by many intermediate filaments. The edges of the focal contact are indicated by the arrowheads. *S*, substrate. Bars, (*C*) 10 μm; (*D–F*) 0.2 μm.

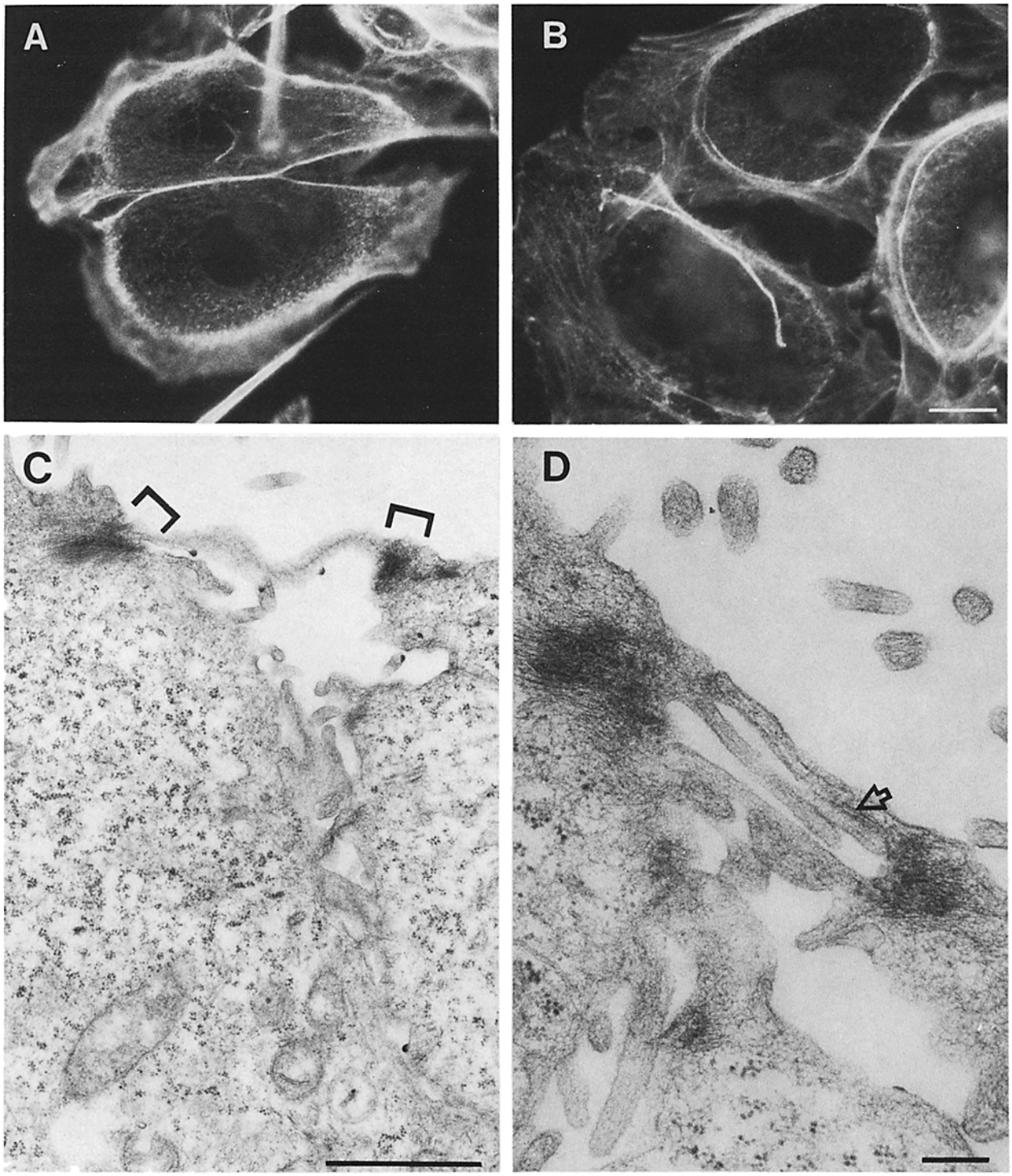


Figure 3. The effect of Ca^{2+} removal on the adherens junction–bound microfilament belt. (*A* and *B*) Localization of actin in MDBK cells that were treated with 2 mM EGTA for either 10 min (*A*) or 30 min (*B*) by labeling with rhodamine–phalloidine. Notice the partial splitting of the actin belt after 10 min and the complete detachment of the belts after 30 min of incubation. Bar, 10 μm. (*C* and *D*) Transmission electron microscopy at low (*C*) and high (*D*) power magnification of MDBK cells, following a 2-min incubation with 4 mM EGTA. Notice the retention of membrane-bound filaments after splitting of the junction (brackets in *C*). Often the two membranes remained attached at positions apical to the adherens junction, near the tight junction area (arrow in *D*). Bars, (*C* and *D*) 1 μm and 0.2 μm, respectively.

the subapical *zonula adhaerens*, closely associated with each other (20); at the ventral cell regions, vinculin is associated with the termini of actin-containing stress fibers (Fig. 5, *A* and *B*; for reviews see references 14 and 20). After detachment of the microfilament bundle from the junctional membrane, vinculin and actin appeared to remain apparently associated, as concluded from the coincident or nearly overlapping fluorescent images (Fig. 5, *C–F*). This association persisted for up

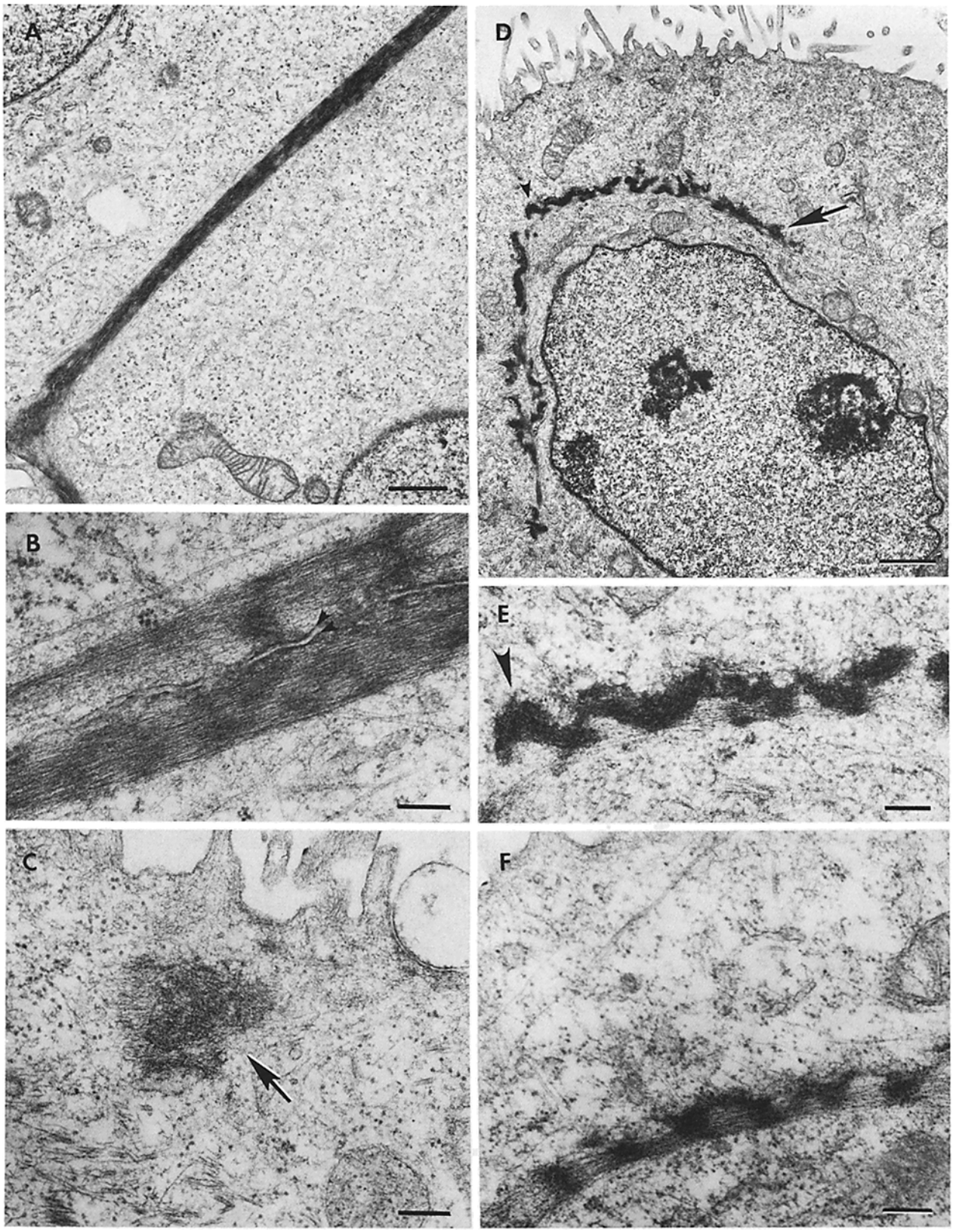


Figure 4. Transmission electron photomicrographs of MDBK cells either incubated in regular medium (*A* and *B*) or 30 min after the addition of 4 mM EGTA (*C–F*). The cells shown in *A*, *B*, and *D–F* were cut parallel to the plane of the substrate; the cell in *C* was cut perpendicularly to the substrate (arrow points to the microfilament belt). (*A*) Survey micrograph showing the continuity of the adherens junction between the two cells (notice the nuclei in upper left and bottom right corners). (*B*) Higher power micrograph showing the close association of the microfilament bundle to the junctional membranes (the latter is indicated by the twin arrowheads). Notice the microtubules which often run along the microfilament belt and the intermediate filaments which apparently associate with it or even penetrate into it. (*C*) A slightly tangential section across the detached filament bundle. (*D* and *E*) A survey and high power photomicrographs showing the convoluted and contracted actin belt at a perinuclear position (arrow in *D*). The arrowheads in *D* and *E* point to the same locations. (*F*) The periodic electron densities of the contracted belt showing typical spacing of 0.3 μm (compared to about ~0.5 μm in the unperturbed belt; see Fig. 1 *E*).

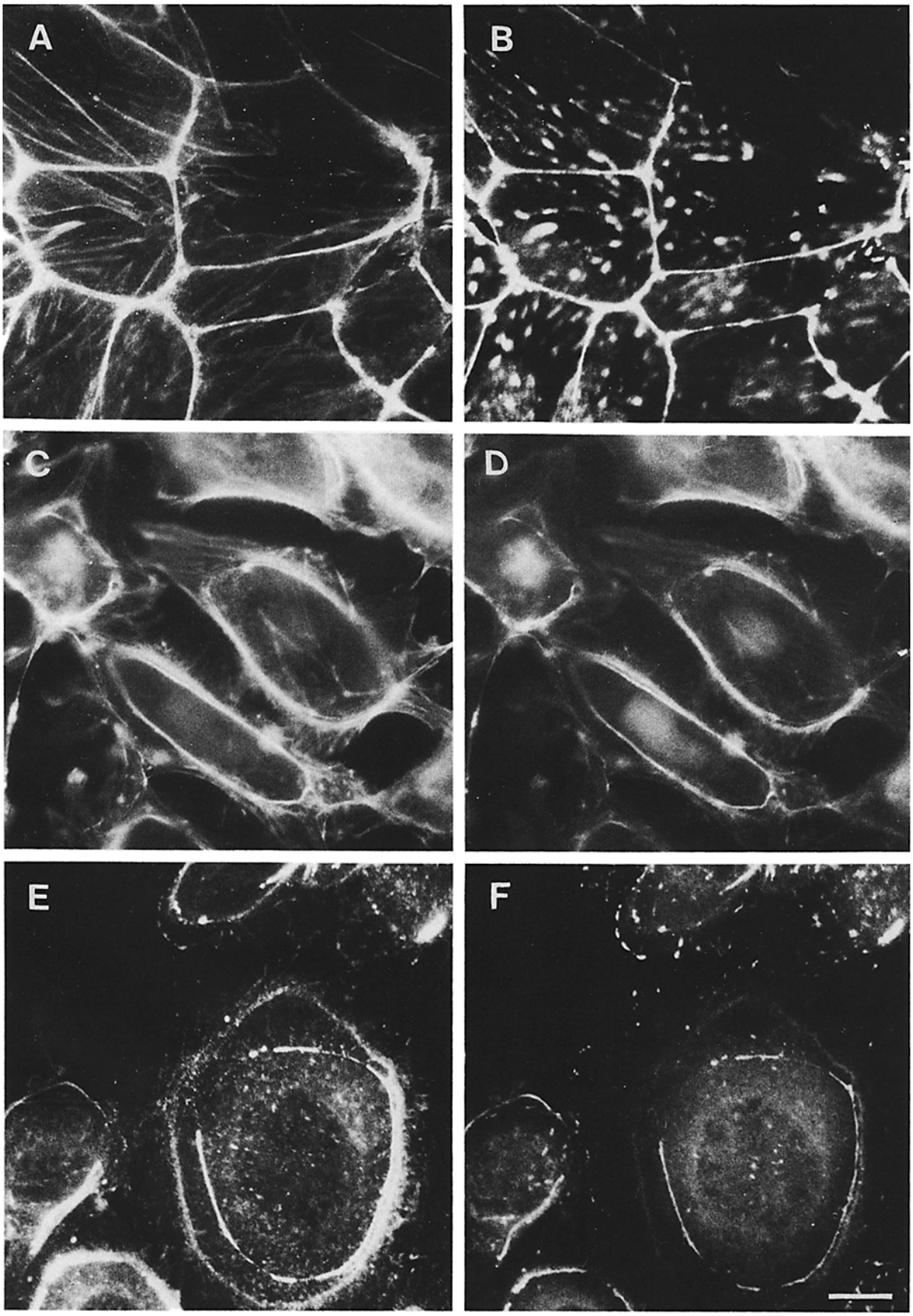


Figure 5. Double labeling of MDBK cells for actin (*A*, *C*, and *E*) using rhodamine–phalloidine, and for vinculin (*B*, *D*, and *F*) using fluorescein-coupled antibodies. The cells were either untreated (*A* and *B*) or incubated with 2 mM EGTA for 30 min (*C* and *D*) or for 2 h (*E* and *F*). Notice the close spatial relationships between actin and vinculin as well as the apparent deterioration of the contracted belt after prolonged incubations. Bar, 10 μm.

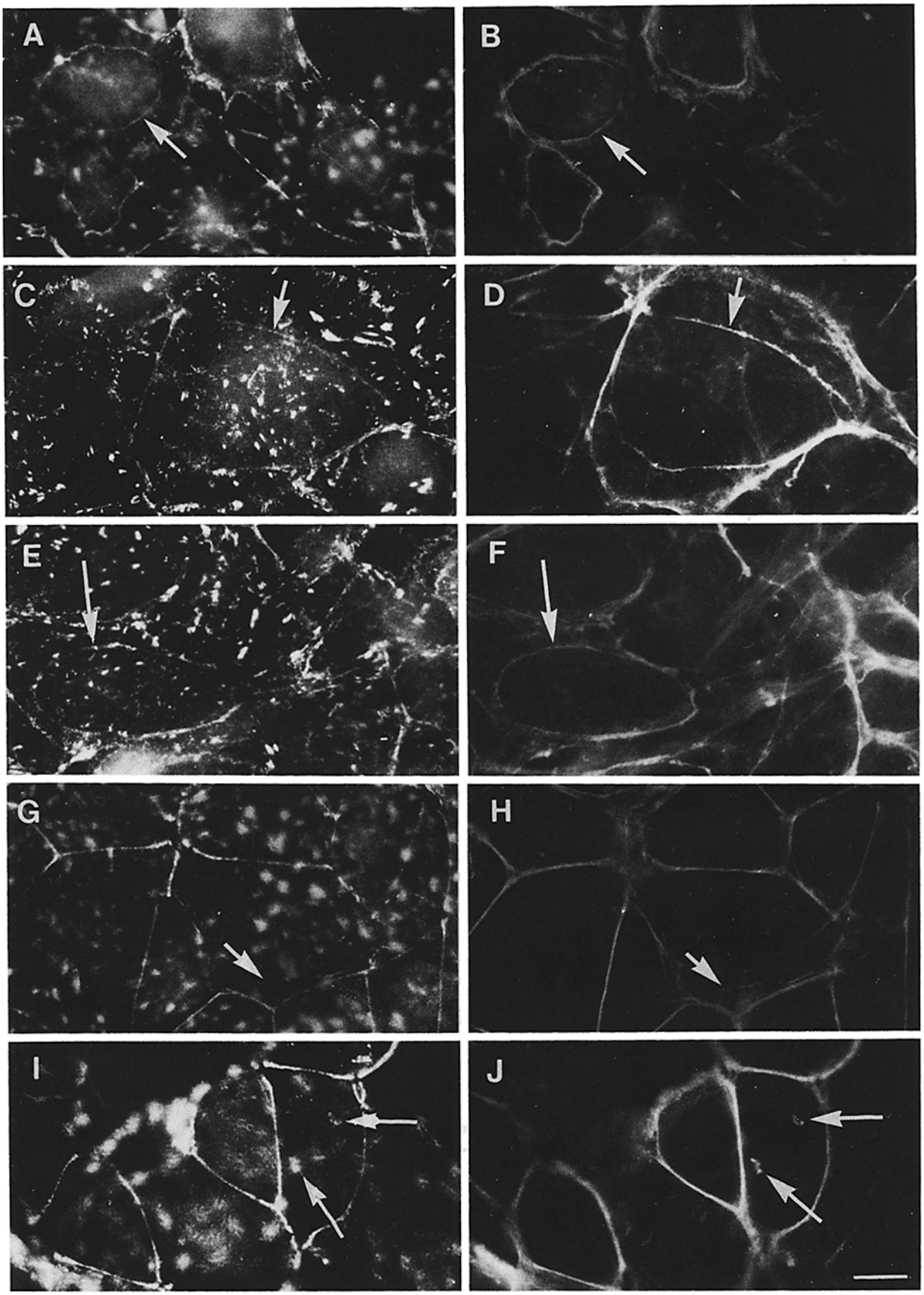


Figure 6. Reformation of junctional actin belt after the addition of fresh medium to EGTA-treated cells. All cells shown here were double labeled for vinculin, rhodamine, and for actin using fluorescein–phalloidin. The labeling for vinculin is shown at the left column (*A*, *C*, *E*, *G*, and *I*), while the labeling of the same cells for actin is shown at the right column (*B*, *D*, *F*, *H*, and *J*). (*A* and *B*) Cells treated with 4 mM EGTA for 30 min (no recovery). (*C–J*) Cells treated for 30 min with 4 mM EGTA and allowed to recover in the presence of fresh medium for 30 min (*C* and *D*); 45 min (*E* and *F*); 90 min (*G* and *H*), and 120 min (*I* and *J*). Notice the detached belts (arrows) which gradually disintegrate concomitantly with the re-establishment of new junctional belts. At times greater than 1 h only faint remains of the detached filaments and the associated vinculin can be seen. Bar, 10 μm.

to 2–3 h, following Ca^{2+} depletion, after which the actin filament bundle with the attached vinculin have undergone a considerable disintegration (Fig. 5, *E–F*). It is noteworthy that cell–substrate focal contacts and the associated vinculin in the cells were far less sensitive to EGTA treatment than the intercellular junctions (this could be assessed by focusing the microscope down to the ventral focal plane), and detachment of focal contacts seemed to be secondary to the chelator-induced rounding-up of the cells (see Discussion).

Reformation of Adherens Junctions in EGTA-treated MDBK Cells after Addition of Ca^{2+} Ions

Cultured MDBK cells treated with 4 mM EGTA for 30 min (after which nearly complete dissociation of their junctions occurred) were supplemented with fresh, Ca^{2+}-containing medium (final Ca^{2+} concentration, ~1.4 mM), and the formation of new adherens junctions examined at different intervals thereafter. When cells showing EGTA-induced detachment of the junctional microfilament belt (vinculin and actin shown in Fig. 6, *A* and *B*) were exposed to fresh, Ca^{2+}-containing medium, belt retraction was not prevented. However, within ~30–45 min after restoration of Ca^{2+} in the medium re-spreading of the cells occurred concomitantly with the formation of new adherens junctions. This was accompanied by the re-appearance of a vinculin- and actin-containing junction-associated filament belt along the new intercellular contacts and by progressive disintegration of the "old" belt in the perinuclear cytoplasm (arrows in Fig. 6). The different stages of this process are depicted by double fluorescent labeling of recovering cells for vinculin and actin at different intervals after Ca^{2+} restoration. It is noteworthy that within 2 h after addition of Ca^{2+}, the reformation of new junction-associated belts was apparently complete, and remains of the contracted belts (still presenting both actin and vinculin) were no longer or only hardly detectable (Fig. 6, *I* and *J*). The process of junction reformation was apparently independent of cellular protein synthesis since the addition of 100 μg/ml cycloheximide to cells during the recovery phase did not inhibit junction formation, nor did it affect the rate of this process (not shown). Similar results were also obtained with cells that had been exposed to 4 mM EGTA for only 2–5 min and then resupplemented with Ca^{2+}-containing medium. This short pre-treatment had only limited, visible effect on the junction-attached microfilament belt. However, the subsequent visible detachment and contraction of the belt and its disintegration took place, irrespective of the restoration of extracellular Ca^{2+} ions. Examination of a large number of MDBK cells at different intervals after addition of Ca^{2+} ions suggested that the "old" belt, once detached, could not re-associate with the plasma membrane and that the assembly of a new junction associated microfilament belt depended on the formation of new intercellular contacts.

Discussion

Studies on the molecular composition of adherens junctions have suggested that these commonly occurring cell contacts consist of several molecular compartments. It was proposed that the establishment of local cell contacts induce the sequential assembly of membrane components and certain intracellular structures as described above (4, 16). Evidence for the existence of different substructures (subdomains) in adherens junctions includes: (*a*) immunoelectron microscopy, showing that vinculin is located closer to the membrane than other microfilament-associated proteins, e.g., α-actinin and tropomyosin (19, 41); (*b*) examination of cells at the initial stages of spreading on planar substrates or during locomotion, indicating that vinculin becomes organized into distinct patches before actin bundles are apparent (15, 21, 35); (*c*) severing of membrane-bound actin by fragmin results in the disappearance of actin and most of its associated proteins but has no comparable effect on the focal contact plaque proteins vinculin and talin (2, 21).

These, as well as additional in vitro studies, have shed some light on the composition and spatial relationships between the two innermost domains of focal contacts, namely the plaque and the associated microfilament bundle. However, only limited information is presently available regarding the interactions between the junctional plaque components and the cytoplasmic faces of the membrane. This is, at least partially, due to difficulties encountered in the selective extraction of vinculin or talin without severely affecting the integrity of the neighboring domains, i.e., the membrane proper and the actin filament bundle. Attempts to study the molecular forces involved in the binding of vinculin to the membrane seem premature in view of the lack of data on the nature of the specific integral transmembrane components.

Our present study has extended previous observations suggesting that the interaction between the junctional plaque of the *zonula adhaerens* and the cytoplasmic surface of the membrane in epithelial cells may be experimentally modulated by the removal of extracellular calcium ions. That study was primarily concerned with the fate of desmosomes which became endocytosed after breakdown of the intercellular junctional complex (32). Using both immunofluorescent microscopy and transmission electron microscopy, we have shown that various elements of the desmosome, including the desmosomal membrane, the plaque, and the cytokeratin filaments, remain associated with each other after breakdown of the junction and endocytosis of the entire desmosomal complex occurs. We have also shown that vinculin-containing material is being translocated from its junction-bound position towards the perinuclear area. In the present study, we have specifically addressed two major questions: (*a*) do the vinculin-containing plaques actually detach after Ca^{2+}-depletion or are they associated with deep invaginations of the plasma membrane?; and (*b*) do cells use the pre-assembled "old" plaques and microfilament bundles for the re-assembly of intercellular junctions after restoration of extracellular Ca^{2+}. To approach these aspects, we have examined the time- and Ca^{2+} concentration–dependence of the cleavage of the junctional complex and the dissociation of structures containing actin and vinculin from the membrane. Specifically, we have demonstrated by electron microscopy that the bundled microfilaments detach as whole large entities from the plasma membranes and do not immediately disperse. This is in contrast to the fate of the desmosomal structures under the same conditions and in the same cells.

We have also found that the detachment of the junctional microfilament belt from the membrane is a largely irreversible process. Thus, once dissociated from the membrane after Ca^{2+} withdrawal, the microfilament belt cannot readily reassociate with the membrane as a whole and will eventually contract and disintegrate, irrespective of whether extracellular

Ca^{2+} is re-supplemented or not. The re-assembly of a new junctional belt is apparently initiated only after the establishment of new intercellular contacts. Taken together, these observations support the previously proposed view that the assembly of adherens junctions is a polar process which is initiated by local membrane contacts and subsequently induces a sequential assembly of the junctional plaque and the microfilament bundle from diffusible subunits (22, 33).

Another aspect that is highlighted by this study is the apparent heterogeneity of adherens-type junctions. In spite of the presence of similar molecular components in intercellular and matrix-attached junctions of this category (i.e., actin and vinculin), the respective responses of the two types of adherens junctions to calcium withdrawal are distinctly different. Unlike the *fascia* and *zonula adhaerens* which are rapidly cleaved in the presence of EGTA, cell–matrix focal contacts exhibit a remarkable stability; at least in MDBK cells, focal contacts remain apparently intact for over 1 h after addition of 2 mM EGTA. The subsequent progressive cell detachment after prolonged incubation with EGTA appears to be secondary to the overall retraction and rounding-up of the cell. Further experiments have indicated that an alternative contact-disrupting agent such as trypsin, instead of EGTA, had quite different effect on MDBK cells, as it rapidly destroyed focal contact–mediated anchorage to the substrate but had only limited and relatively slow effects on the intercellular junctions, commonly resulting in the release of large, apparently intact epithelial sheets from the substrate (data not shown). Moreover, recent studies on the molecular constituents of the two types of adherens-type cell contacts have pointed to significant differences. Intercellular adherens contacts of lens, cardiac muscle, and various cultured cells are associated with a recently described membrane protein of M_r 135,000 (23, 43) that has not been detected in focal contacts or in other cell matrix contacts. Furthermore, the junctional plaques associated with contacts to noncellular matrices have been reported to contain talin, whereas this protein is absent from intercellular junctions (23).

Another aspect of the present study is related to the involvement of Ca^{2+} in the induction of adherens-junction formation and the subsequent maintenance of those junctions. Over the last few years, many studies have pointed to the involvement of Ca^{2+} ions in cell adhesion in a variety of systems. This was often manifested by the requirement for Ca^{2+} ions for the formation of visible cell contacts. Only recently, significant progress has been made in the elucidation of the molecular basis for Ca^{2+}-dependent (and independent) cell adhesion. This is manifested by the identification of a family of "contact receptors" or "cell adhesion molecules" (9, 13, 28, 44). While most of these molecules have not been localized so far to a defined type of cell junction it has been reported that uvomorulin (3), also known as L-CAM or E-cadherin, is specifically enriched in *zonulae adherentia*. Our experience, however, indicates that the M_r 135,000 protein is present in cells from which uvomorulin is apparently absent and its presence in diverse adherens junctions in heart, lens, kidneys, etc., is most prominent. Additional studies on the 135-kD protein and its Ca^{2+} dependence are now in progress.

As far as the physiological effect of Ca^{2+} withdrawal is concerned, it has often been argued that the primary target affected by Ca^{2+} withdrawal is the tight junction and that the apparent increase in transepithelial permeability and cell depolarization induced by Ca^{2+} chelators is due to the disruption of this particular junctional element (i.e., 7, 12, 36, 39). In view of the changes which occur at the *zonula adhaerens* and desmosomes after removal of extracellular Ca^{2+} and the relative resistance of tight junctions (see Fig. 3 *D*), it seems now necessary to re-evaluate the differential involvement of the various junctional elements in the establishment of epithelial cell polarity. This is especially relevant to adherens junctions which, in polar epithelial cells, form a continuous belt in the subapical region, just like the tight junctions do.

The direct molecular basis for Ca^{2+} involvement in the formation and maintenance of intercellular adherens junctions is still not clear. One possibility is that Ca^{2+} ions directly act as intercellular linkers, bridging between surface receptors of the two cells in contact. Alternatively, Ca^{2+} ions may bind to junctional surface components and affect their binding capacity either allosterically or by the induction of their lateral aggregation at the plane of the membrane. A third mechanism which may be considered whereby EGTA causes depletion of Ca^{2+} ions from intracellular pools does not seem likely in view of the following findings: (*a*) the visible effect of EGTA is relatively rapid; (*b*) addition of Ca^{2+} ionophore together with EGTA did not facilitate the process (data not shown); and (*c*) free Ca^{2+} concentration in the culture medium, even after addition of effective concentrations of EGTA, is still higher than that found in the cytoplasm. Whatever the exact mechanism of Ca^{2+} action is, the present study indicates that the *zonula adhaerens* is one of the primary sites of stable, Ca^{2+}-mediated cell contact. Furthermore, a direct examination of Ca^{2+} binding properties of specific junctional proteins might shed light on basic mechanisms of intercellular interaction and communication, and on the involvement of Ca^{2+} ions in these processes.

We would like to acknowledge with much gratitude the excellent technical assistance of Mrs. E. Sabanai in the electron microscopy.

The study was supported by grants from the Muscular Dystrophy Association and from the Erna Rothstein Foundation.

Received for publication 19 November 1985, and in revised form 17 January 1986.

References

1. Abercrombie, M., and G. A. Dunn. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp. Cell Res.* 92:57–62.

2. Avnur, Z., J. V. Small, and B. Geiger. 1983. Actin-independent association of vinculin with the cytoplasmic aspect of the plasma membrane in cell contact areas. *J. Cell Biol.* 96: 1622–1630.

3. Boller, K., D. Vestweber, and R. Kemler. 1985. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions in adult intestinal epithelial cells. *J. Cell Biol.* 100:327–332.

4. Burridge, K., and J. Feramisco. 1980. Microinjection and localization of a 130 K protein in living fibroblasts: a relationship to actin and fibronectin. Cell. 19:587–595.

5. Burridge, K., and L. Connell. 1983. A new protein of adhesion plaques and ruffling membranes. *J. Cell Biol.* 97:359–367.

6. Burridge, K., and L. Connell. 1983. Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin–membrane interaction. *Cell Motility.* 3:405–417.

7. Cereijido, M., I. Meza, and A. Martinez-Palono. 1981. Occluding junctions in cultured epithelial monolayers. *Am. J. Physiol.* 9:C96–C102.

8. Curtis, A. S. G. 1964. The mechanism of adhesion cells to glass. A study by interference reflection microscopy. *J. Cell Biol.* 20:199–215.

9. Damsky, C. H., J. Richa, D. Solter, K. Knudsen, and C. A. Buck. 1983. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell.* 34:455–466.

10. Farquhar, M. G., and G. E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375–409.

11. Fawcett, D. W. 1981. The Cell. 2nd edition. Saunders Company, Phila-

delphia, p. 1–862.

12. Galli, P., A. Brenna, P. De-Camilli, and J. Meldolesi. 1976. Extracellular calcium and the organization of tight junctions in pancreatic acinar cells. *Exp. Cell Res.* 99:178–183.

13. Gallin, W. J., G. M. Edelman, and B. A. Cunningham. 1983. Characterization of L-Cam, a major cell adhesion molecule from embryonic liver cells. *Proc. Natl. Acad. Sci. USA.* 80:1038–1042.

14. Geiger, B. 1979. A 130 K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193–205.

15. Geiger, B. 1981. Transmembrane-linkage and cell attachment: the role of vinculin. *In* International Cell Biology 1980–1981. H. G. Schweiger, editor. Springer-Verlag. 761–773.

16. Geiger, B. 1982. Involvement of vinculin in contact-induced cytoskeletal interaction. *Cold Spring Harbor Symp. Quant. Biol.* 46:671–682.

17. Geiger, B. 1983. Membrane cytoskeleton interaction. *Biochim. Biophys. Acta* 737:305–341.

18. Geiger, B., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membrane. *Proc. Natl. Acad. Sci. USA.* 77:4127–4131.

19. Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscopic studies of membrane-microfilament interaction. The distribution of α-actinin, tropomyosin, and vinculin in intestinal epithelial brush border and in chicken gizzard smooth muscle. *J. Cell Biol.* 91:614–628.

20. Geiger, B., E. Schmid, and W. W. Franke. 1983. Spatial distribution of proteins specific for desmosomes and *adhaerens* junctions in epithelial cells demonstrated by double immunofluorescence microscopy. *Differentiation.* 23:189–205.

21. Geiger, B., Z. Avnur, G. Rinnerthaler, H. Hinssen, and V. J. Small. 1984. Microfilament organizing centers in areas of cell contact cytoskeletal-interactions during cell attachment and locomotion. *J. Cell Biol.* 99 (1, Pt. 2):83s–91s.

22. Geiger, B., Z. Avnur, T. E. Kreis, and J. Schlessinger. 1984. The dynamics of cytoskeletal organization in areas of cell contact. *In* Cell and Muscle Motility, Vol. 5. J. W. Shay, editor. Plenum Publishing Corp., New York. 195–234.

23. Geiger, B., T. Volk, and T. Volberg. 1985. Molecular heterogeneity of adherens junctions. *J. Cell Biol.* 101:1523–1531.

24. Geiger, B., Z. Avnur, T. Volberg, and T. Volk. 1985. Molecular domains of adherens junctions. *In* The Cell in Contact. Edelman, G. M., and J.-P. Thiery, editors. Neurosc. Inst. Pub. 461–490.

25. Grinnel, F. 1978. Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* 53:65–144.

26. Heaysman, J. E. M., and S. M. Pegrum. 1973. Early contacts between fibroblasts. An ultra-structural study. *Exp. Cell Res.* 78:71–78.

27. Heaysman, J. E. M., and S. M. Pegrum. 1982. Early cell contacts in culture. *In* Cell Behaviours. R. Bellair, A. Curtis, and G. Dunn, editors. Cambridge University Press. 49–76.

28. Hyafil, F., C. Babinet, and F. Jacob. 1981. Cell–cell interactions in early embryogenesis: a molecular approach to the role of calcium. *Cell.* 26:447–454.

29. Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts. An interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129–159.

30. Izzard, C. S., and L. R. Lochner. 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J. Cell Sci.* 42:81–116.

31. Jones, J. C. R., A. E. Goldman, P. M. Steinert, S. Yuspa, and R. D. Goldman. 1982. Dynamic aspects of the supermolecular organization of intermediate filament networks in cultured epidermal cells. *Cell Motility.* 2:197–213.

32. Kartenbeck, J., E. Schmid, W. W. Franke, and B. Geiger. 1982. Different modes of internalization of proteins associated with adherens junctions and desmosomes: experimental separation of lateral contacts induces endocytosis of desmosomal plaque material. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:725–732.

33. Kreis, T. E., Z. Avnur, J. Schlessinger, and B. Geiger. 1985. Dynamic properties of cytoskeletal proteins in focal contacts. *In* Molecular Biology of the Cytoskeleton. Cold Spring Harbor Laboratories, New York. 45–47.

34. Lazarides, E., and K. Burridge. 1975. α-Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289–298.

35. Lehtonen, E., V.-P. Lehto, R. A. Badely, and I. Virtanen. 1983. Formation of vinculin plaques precedes other cytoskeletal changes during differentiation. *Exp. Cell Res.* 144:182–190.

36. Palant, C. E., M. E. Duffey, B. K. Mookirjee, S. Ho, and C. J. Butzel. 1981. Ca^{2+} regulation of tight junction permeability and structure in *Necturus* gallbladder. *Am. J. Physiol.* 245:C203–C212.

37. Palay, S. L. 1967. Principles of cellular organization in the nervous system. *In* The Neurosciences: A Study Program. G. C. Quarton, T. Melnechuk, and F. O. Schmitt, editors. Rockefeller University Press, New York. 24–31.

38. Philip, N. J., and V. T. Nachmias. 1985. Components of the cytoskeleton in the retinal pigmented epithelium of the chick. *J. Cell Biol.* 101:358–362.

39. Pitelka, D. R., B. N. Taggart, and S. T. Hamamoto. 1983. Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary cells in culture. *J. Cell Biol.* 96:611–624.

40. Staehelin, A. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* 39:191–283.

41. Tokuyasu, K. T., A. H. Dutton, B. Geiger, and S. J. Singer. 1981. Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy. *Proc. Natl. Acad. Sci. USA.* 78:7619–7623.

42. Lehto, V-P., and I. Virtanen. 1985. Vinculin in cultured bovine lens-forming cells. *Cell Differentiation.* 16:153–160.

43. Volk, T., and B. Geiger. 1984. A new 135 kd membrane protein of intercellular adherens junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2249–2260.

44. Yoshida-Noro, C., N. Suzuki, and M. Takeichi. 1984. Molecular nature of the calcium-dependent cell–cell adhesion system in mouse tetratocarcinoma and embryonic cells studied with monoclonal antibody. *Dev. Biol.* 101:19–27.