Calcium-induced Assembly of Adherens Junctions in Keratinocytes

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Abstract. Extracellular calcium concentration has been shown to control the stratification of cultured keratinocytes, presumably by regulation of formation of desmosomes. Previous studies have shown that keratinocytes cultured in medium containing 0.1 mM Ca^{++} form loose colonies without desmosomes. If the Ca^{++} is raised to 1 mM, desmosomes are assembled and the distribution of keratin filaments is altered. We have examined the disposition of vinculin and actin in keratinocytes under similar conditions. Using immunofluorescence microscopy we show that raising $[Ca^{++}]$ in the medium dramatically alters the distribution of vinculin and actin and results in the formation of adherens-type junctions within 15 min after switching to high calcium medium. Borders of cells at the edge of colonies, which are not proximal to other cells, are not affected, while cells in the interior of the colony form junctions around their periphery. Attachment plaques in keratinocytes grown in low calcium medium are located at the ventral plane of the cell, but junctions formed after switching to high calcium are not, as demonstrated by interference reflection microscopy. In cells colabeled with antibodies against vinculin and desmoplakin, vinculin-containing adherens junctions were visible before desmosomal junctions when cells were switched to high calcium. Although newly formed vinculin-containing structures in high calcium cells, like desmosomes, colocalize with phase-dense structures, superimposition of video fluorescence images using digitized fluorescence microscopy indicates that adherens junctions and desmosomes are discrete structures. Adherens junctions, like desmosomes, may play an essential role in controlling stratification of keratinocytes.

DESMOSOMES, major junctional structures in epidermis, are readily formed in epithelial cell cultures. Growth of cultured keratinocytes at reduced concentrations of Ca^{++} (0.1 mM or less) prevents formation of desmosomes; the cells fail to stratify but rather continue to grow as a monolayer (Hennings et al., 1980). Raising the Ca^{++} of the medium induces assembly of desmosomes (Hennings and Holbrook, 1983), produces striking alterations in the distribution of several desmosomal proteins and of keratin filaments (Watt et al., 1984; Jones and Goldman, 1985) to which the desmosomes attach (Jones and Goldman, 1985), and is associated with stratification of the keratinocytes.

A second type of junctional structure, the adherens junction (see Farquhar and Palade, 1963; McNutt and Weinstein, 1973; and Staehelin, 1974, for review of terminology), best described in intestinal epithelium, is associated with vinculin, alpha-actinin, and actin, but not with desmosomal proteins or keratins. In intestinal epithelium the adherens junction is part of a junctional complex comprising the apically located tight junction (zonula occludens), the intermediate or adherens junction (zonula adherens), also called a "belt desmosome," which forms a beltlike structure just below the tight junction, and the desmosome (macula adherens), also called a "spot" desmosome. In current use the term adherens junction refers to the zonula adherens, which is associated with microfilament bundles containing actin, unlike the desmosome, which is associated with keratin filaments.

In epithelial cells in culture, vinculin, actin, and alpha-actinin are found in three different structures. The first is the cell-to-substrate focal contact, also called the attachment plaque, which is located at the ventral aspect of the cell and also occurs in fibroblasts (Geiger, 1979; Burridge and Feramisco, 1980; Geiger et al., 1980), and which is the point of insertion of microfilament bundles at the plasma membrane (Abercrombie, 1971; Geiger, 1979, 1983; Burridge and Feramisco, 1980). The second is the classical beltlike zonula adherens (Farquhar and Palade, 1963; Staehelin, 1974; Geiger et al., 1981). Last, in addition to this well described peripheral belt structure, discontinuous structures resembling desmosomes in morphology but associated with 70-Å filaments containing actin rather than with 100-Å keratin filaments have also been described under different names by different authors (McNutt and Weinstein, 1973; Staehelin, 1974; Geiger et al., 1983; Drenckhahn and Franz, 1986). The physical relationship of desmosomes to these adherens-type junctions varies in different tissues and in different types of cells in culture (Geiger et al., 1983; Drenckhahn and Franz, 1986) and has not been described in keratinocytes.

We have examined the effect of elevating Ca^{++} on certain of the proteins of adherens junctions in keratinocytes. Using antibodies against vinculin, we have found that keratinocytes maintained in low calcium demonstrate typical vinculin-containing focal contacts; vinculin is dramatically altered in its location (moving to the cell periphery) within minutes af-

ter Ca^{++} is raised and forms junctional structures (adherens junctions). Although desmosomes can be demonstrated near these structures, the two types of junctions are distinct. In addition, the distribution of actin, which exists as diffusely distributed stress fibers in low calcium, is also altered, and forms more tightly bundled peripheral belts.

Materials and Methods

Cell Culture

Keratinocytes were obtained from newborn foreskins and initiated into culture by a modification (O'Keefe et al., 1982) of the method of Rheinwald and Green (1977) and then subcultured in MCDB 153 according to Boyce and Ham (1983) with 0.1 mM Ca^{++}. Medium was supplemented with hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), epidermal growth factor (5 ng/ml), and bovine pituitary extract (150 μg/ml) (Boyce and Ham, 1983) and with supplemental essential amino acids (histidine, 2×10^{-4} M; isoleucine, 7.5×10^{-4} M; methionine, 9×10^{-5} M; phenylalanine, 9×10^{-5} M; tryptophan, 4.5×10^{-5} M; and tyrosine, 7.5×10^{-5} M). Cells were subcultured at 5,000 cells per cm^2 as previously described (O'Keefe et al., 1985).

Immunofluorescence

Cells plated on glass 12-mm round coverslips were fixed in 3.7% formaldehyde in PBS containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, pH 7.4, for 5 min, and extracted in 0.1% Triton X-100 for 5 min at 24°C as previously described (Herman and Pledger, 1985). The coverslips were incubated with primary antibodies for 30 min at 37°C, washed in PBS, incubated with fluorescein-conjugated second antibody containing 5 μl rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) per 100 μl IgG, washed, and mounted in Gelvatol (Monsanto Co., St. Louis, MO). Rabbit antibody to chicken vinculin (purified according to Feramisco and Burridge [1980]) labeled attachment plaques in cultured cells from human, mouse, and monkey and immunoprecipitated a single 130-kD band in cultures of keratinocytes biosynthetically labeled with [^{35}S]methionine in comparison with nonimmune rabbit serum (data not shown). Guinea pig antivinculin was a gift of K. Burridge (University of North Carolina). Antidesmoplakin antibody was a kind gift of D. Drenckhahn (University of Marburg, Marburg, Federal Republic of Germany). Coverslips were viewed in a Leitz epifluorescence microscope (63× 1.4 NA Neofluor oil immersion lens) equipped with appropriate filters to view either fluorescein- or rhodamine-labeled structures, photographed with Kodak Tri-X film at an ASA of 1,600, and developed in Diafine (Acufine, Inc., Chicago, IL).

Digitized Fluorescence and Interference Contrast Microscopy

Digitized video images of keratinocytes stained for the demonstration of vinculin, desmoplakin, or actin were obtained using a digitized low light level microscope system, the design of which has been previously described (DiGuiseppi et al., 1985). Images were magnified three times from the microscope (63× 1.4 NA Neofluor lens) with a zoom rifle scope, collected using a camera (ISIT-66; Dage-MTI Inc., Wabash, MI), and digitized with a board set (IP-512 series; Imaging Technologies, Woburn, MA) housed in an 11/23 Q bus-based microcomputer (Digital Equipment Corp., Marlborough, MA). The digitization process results in an image containing 65,536 discrete points (256 × 256 matrix). After thresholding to remove background and out of focus fluorescence, we pseudocolored the antivinculin image green and the antidesmoplakin image red. The antivinculin image was then overlaid onto the antidesmoplakin image. The result of this process was that if the same pixel in both images contained information (intensity) the observed color would be yellow (red on green), whereas if pixels in only one of the two original images contained information the color would remain unchanged (either red or green). Photographs were obtained using Ektachrome 100 ASA film and line drawings were made from projections to demonstrate potential areas of overlap between vinculin and desmoplakin.

Focal contacts (adhesion plaques) were visualized by interference contrast microscopy. Excitation illumination passes from a 100-W Hg lamp through a 546 nm (±5 nm full width half-maximum) interference filter, then through a polarizer and is reflected onto the specimen by an H-PL-POL reflector (Zeiss, Oberkochen, Federal Republic of Germany). The light passes through a 63× antiflex objective (containing a quarter wave plate), strikes the specimen, is reflected back into the objective, and passes through an analyzer. The image is recorded with the video camera and computer system or photographed. The use of epiillumination allows visualization of antivinculin staining and focal contacts in the same cell.

Electron Microscopy

Cells grown on coverslips were fixed in half-strength Karnovsky's fixative, postfixed in osmium tetroxide, embedded in Polybed 812 (Polysciences Inc., Warrington, PA), stained with uranyl acetate and lead acetate, and processed and examined in a JEM 100B electron microscope.

Results

Calcium-induced Alteration in the Location of Vinculin

Keratinocytes grown in medium with 0.1 mM Ca^{++} have refractile borders on phase-contrast microscopy and grow in a loose colonial morphology (Fig. 1 *a*). After a 15-min exposure to medium containing 1.0 mM Ca^{++}, borders have begun to fuse (Fig. 1 *b*). From this time on the cells associate more closely (Figs. 1 *c* and 2 *h*), and by 20 h they begin to overlap and stratify (Fig. 1 *d*) and approximate a stratified squamous epithelium.

Cells grown in low concentrations of Ca^{++} (0.1 mM or less), fixed, and stained for vinculin revealed structures typical of attachment plaques (Fig. 2 *a*) located at the ventral aspect of the cell. These cells formed few apparent junctions with adjacent cells but formed colonies in which adjacent cells sometimes overlapped one another without forming vinculin-staining junctional structures.

Within 15 min of addition of Ca^{++} to a final concentration of 1 mM, a marked change in vinculin staining was apparent. In colonies of cells in which cell borders were in proximity, the vinculin-containing plaques first moved to the cell periphery, sometimes before the borders of adjacent cells had met (Fig. 2 *b*, *arrow*). As the cells developed contacts, vinculin became concentrated at junctional areas between cells. At the same time, staining of typical attachment plaques at the ventral surface became weaker or disappeared, and granular cytoplasmic fluorescence, presumably from soluble cytoplasmic vinculin (Rosenfield et al., 1985) often increased (Fig. 2 *c*, 15 min; Fig. 2 *d*, *e*, 2 h). By 15 min to 1 h after addition of Ca^{++}, depending on the proximity of the cells forming junctions, formation of vinculin-staining junctions appeared to be complete. Closely apposed cells formed junctions promptly, but cells in proximity with some space between them showed deposition of vinculin at their extreme borders, as in Fig. 2 *b*, still in attachment plaques, and only formed junctions after the cells established contact. The appearance of basally situated vinculin at the extreme periphery of the cell in adjacent cells with slight separations (Fig. 2 *b*) was not an artifact of fixation, since it was not present after longer exposures to high Ca^{++} and was occasionally present in isolated cells. This vinculin was removed when the cells established contact. Further formation of junctions continued as more cells became contiguous; consolidation of the colony then followed.

In cells with a free border at the edge of the colony, cell borders not in contact with other cells continued to show apparently unchanged vinculin attachment plaque staining at the basal aspect of the cell (Fig. 2 *c*, *arrow*). Cells in colonies

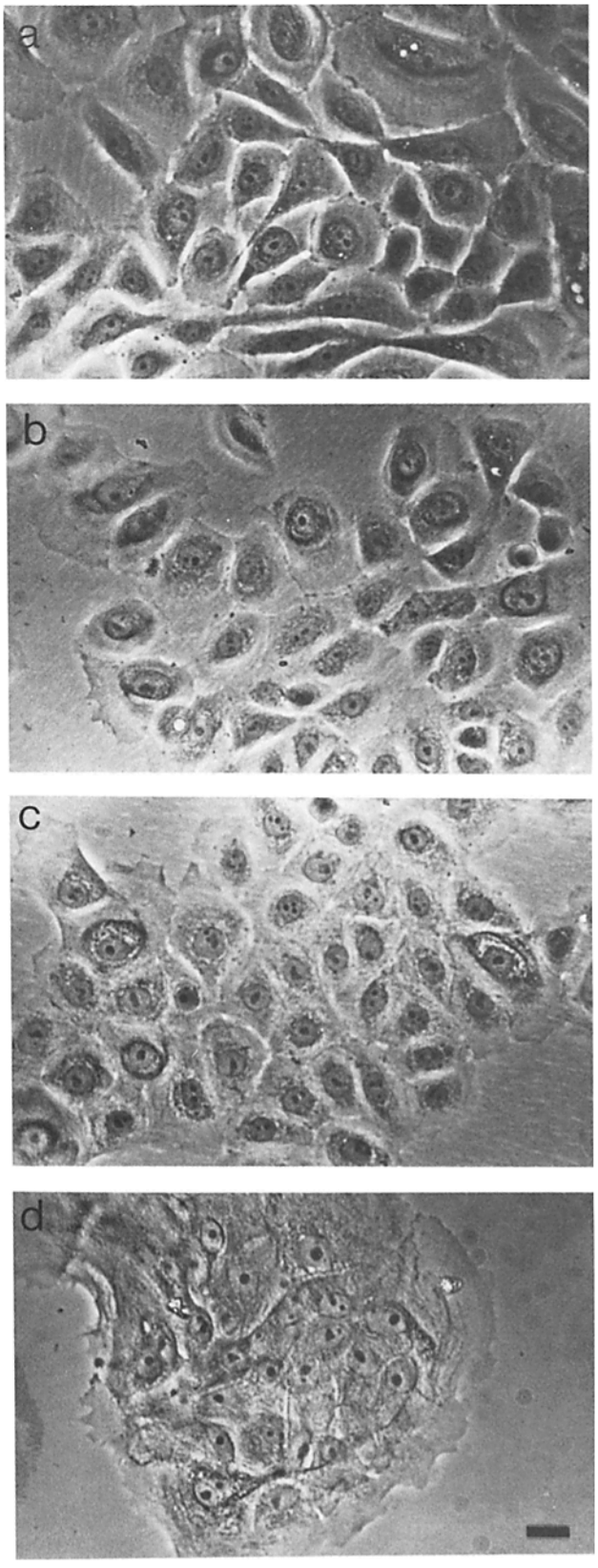


Figure 1. Phase-contrast images of keratinocyte colonies before and after Ca^{++} switch. Cells were photographed before (*a*) or after 15 (*b*) or 120 (*c*) min or 20 h (*d*) incubation in medium containing 1 mM Ca^{++}. Control cells show refractile borders, and regions where cells meet are readily apparent in 0.1 mM Ca^{++}. After the switch the cell borders are less distinct and some phase-dense junctions are present (*b* and *c*). After 20 h stratification has occurred (cells are seen to overlap). Bar, 20 μm.

with neighboring cells on all sides lost attachment plaque staining entirely and developed junctional staining (Fig. 2 *d*). In some cells parallel pairs of vinculin-containing junctions were noted (Fig. 2, *e–g*); this appearance may have resulted from fortuitous viewing between apposed junctions along an axis perpendicular to the plane of the cells. This staining pattern seemed to be associated with compact colonies rather than with loosely associated cells and was present both in the interior of colonies and also at their borders. This type of staining did not develop until later stages in adherens junction assembly. Parallel arrays of vinculin-staining structures have been described in other cultured epithelial cells (Shriver and Rohrschneider, 1981).

To examine the relationship of actin-containing fibers to adhesion plaques and junctions, we performed double immunofluorescence using fluorescein (Fig. 3, *left*, vinculin staining) and rhodamine (Fig. 3, *right*, actin staining). The distribution of microfilaments in the cells exposed to 1 mM Ca^{++} was also altered, apparently in concert with changes in vinculin. At 0.1 mM Ca^{++}, actin-containing fibers in cells stained for vinculin were scattered diffusely throughout the cytoplasm aligned with the longer axis of the cell (Fig. 3, *a* and *b*) or in small groups of shorter bundles (Fig. 3, *c* and *d*). In some cases the ends of short groups of actin fibers could be seen to originate in attachment plaques, as demonstrated by their staining for vinculin (Fig. 3, *c* and *d*, *arrows*). In contrast, in cells exposed to 1 mM Ca^{++} for 2 h, actin fibers formed peripheral belts either in very compact bundles (Fig. 3, *e* and *f*) or in loosely grouped bundles in which individual fibers were still visible (Fig. 3, *g* and *h*). At the final stages of formation of the junctions, stress fibers from adjoining cells appeared to meet at the junction, and punctate actin staining was present at the site of vinculin-containing junctions (Fig. 3, *g* and *h*). Unlike stress fibers in cells in 0.1 mM Ca^{++}, those in cells treated with 1.0 mM Ca^{++} were frequently absent over the nucleus.

In some cases, junctional vinculin-containing structures began to form before all attachment plaques had disappeared. As shown in Fig. 3, *i* and *j*, both attachment plaques and vinculin-containing junctions can be present simultaneously, showing that the junctions can form before the attachment plaques are removed. At this stage in the formation of intercellular junctions, in this case after 1 h in 1.0 mM Ca^{++}, actin-containing fibers appeared to extend from the interior of both adjoining cells directly to the junction (identified by vinculin staining) and often gave the appearance of spanning the space between the cells (Fig. 3, *i* and *j*).

Interference Reflection Studies of Vinculin-staining Structures

To determine whether structures staining positively for vinculin were located at the basal aspect of the cell under conditions of high and low Ca^{++}, we examined cells both by immunofluorescence and by interference reflection microscopy. At 0.1 mM Ca^{++} (Fig. 4, *a* and *b*), antivinculin staining adhesion plaques that appeared to be in focus at the ventral aspect of the cell (Fig. 4 *a*) correspond to dark areas visible under interference reflection (Fig. 4 *b*). In contrast, junctional structures (present after treatment of cultures with 1.0

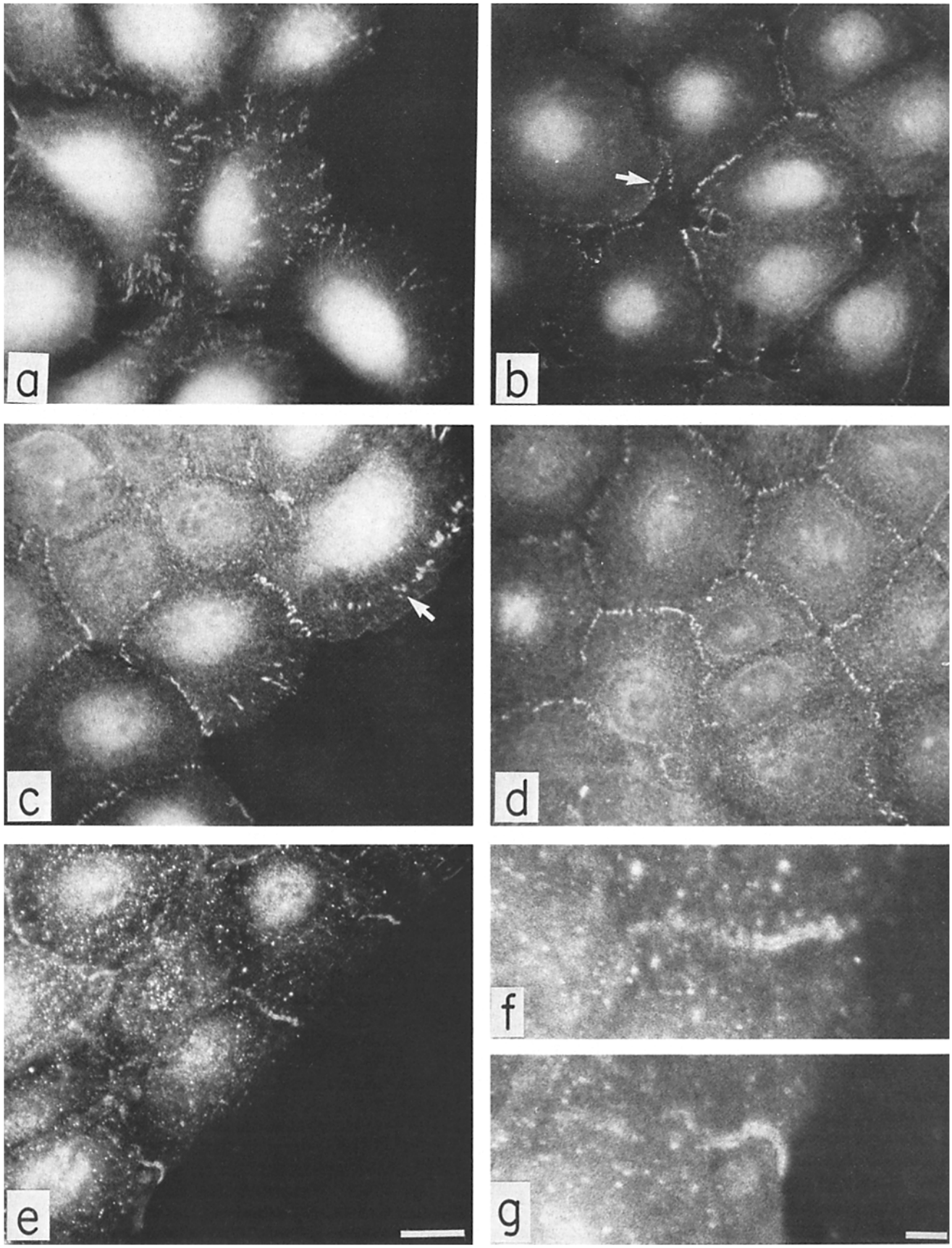


Figure 2. Effect of switching from low (0.1 mM) to high (1.0 mM) Ca^{++} on vinculin-containing structures in keratinocytes. Cells were grown on glass coverslips in 0.1 mM Ca^{++} and treated with or without 1.0 mM Ca^{++} before being fixed, extracted, and stained for vinculin. Cells in 0.1 mM Ca^{++} (*a*), and 15 min after change to 1.0 mM Ca^{++} (*b* and *c*). Shortly after raising the Ca^{++} in the medium, attachment

mM Ca^{++} for 1 h) that contained vinculin by immunofluorescence (Fig. 4 *c*) did not correspond with dark areas (Fig. 4 *d*) and therefore were located further than 100 Å from the ventral plane of the cell. Focusing also confirmed the impression that junctional vinculin was not ventrally situated. In colonies of cells treated for 1 h with 1 mM Ca^{++}, remaining peripheral attachment plaques were visible at the basal aspects of the cells (Fig. 4 *e*), but focusing at a higher plane in the same colony (Fig. 4 *f*) brought the vinculin-staining junctional structures into focus.

Visualization of Junctions under Phase-Contrast Microscopy and Comparison with Desmosomes

Junctions between keratinocytes have previously been visualized by immunofluorescence using antibodies to desmoplakins and cytokeratins after increasing medium Ca^{++}. Assembly of desmosomes was observed to begin within 15 min and was complete after 2 h (Watt et al., 1984; Jones and Goldman, 1985), and the desmosomes observed by immunofluorescence appeared to correspond in position with phase-dense structures (Jones and Goldman, 1985). To compare the relationship of phase-dense structures, desmosomes, and vinculin-containing junctions in keratinocytes, we compared phase and fluorescence images of cells stained for vinculin, desmoplakin, and actin. Vinculin-staining junctions (Fig. 5, *b* and *e*) were superimposable on the phase-dense areas (Fig. 5, *a* and *d*) demonstrated under phase-contrast microscopy and also coincided with junctional actin staining (cf. Fig. 5 *c*). Since these phase-dense areas between keratinocytes have been previously shown (Jones and Goldman, 1985) to correlate with staining of desmosomes, we compared the phase-dense regions with desmosomal staining and confirmed that the phase-dense regions were similar in position to desmosomal staining in position in our cultures (Fig. 5, *f* and *g*). Since phase-dense regions between cells corresponded with the location of both the adherens junctions and desmosomes, our results show that both the adherens junctions and desmosomes are present in regions where intercellular junctions formed.

To more precisely define the relationship of adherens junctions to desmosomes we performed double immunofluorescence using rabbit antidesmosomal antibody and guinea pig antivinculin antibody. The results showed that both vinculin-containing junctions (Fig. 6 *a*) and desmosomes (Fig. 6 *b*) were present at regions where cells joined. To further specify the interrelationship between these structures we magnified the fluorescence images of vinculin-containing and desmosomal structures (from the same cell) using digitized fluorescence microscopy (DiGuiseppi et al., 1985). The digitized images consist of a spatial map (matrix) of numerical values representing different intensities of fluorescence. To compare the location of junctional vinculin and desmosome-staining junctional structures in the two images we measured the nonjunctional fluorescence intensity and set all pixels with values equal to or below the nonjunctional intensity to zero (by altering the threshold for visible fluorescence on the computer); this process also removes background and out of focus fluorescence. The two images were then superimposed; the result is shown in Fig. 6. The original video images of cells stained for vinculin (Fig. 6 *a*) and desmosomes (Fig. 6 *b*), the corrected images of vinculin (Fig. 6 *c*) or desmosomal (Fig. 6 *d*) structures, and the overlaid image (Fig. 6 *e*) of the relationship of the two junctions are shown. A second overlaid image is shown in Fig. 6 *f*.

Although a few areas in the superimposed image contain structures staining for both junctional vinculin and desmosomes (Fig. 6 *e* and *f*, *black areas*), the two types of staining could be clearly distinguished in most areas. Since this two-dimensional image may include some overlap of structures that would be found to be discrete if viewed in three dimensions, some degree of overlap in the composite is to be expected. Further comparison of the staining patterns of actin, known to be associated with adherens junctions, and desmosomes, associated with keratin filaments, indicated that there appeared to be no relationship between the two (data not shown). We conclude that the adherens junctions and desmosomes that formed in keratinocytes in response to elevation of Ca^{++}, although situated in similar regions of the cell and in proximity, are discrete junctional structures.

Electron microscopy confirmed the presence of structures characteristic of adherens junctions. Control cells in 0.1 mM Ca^{++} had no adherens junctions or desmosomes, but junctions were present in cells switched to 1 mM Ca^{++} (Fig. 7). After 20 h, when the cells had stratified, numerous desmosomes and adherens junctions were present.

Discussion

We have found that keratinocytes cultured in 0.1 mM Ca^{++} and then exposed to physiologic Ca^{++} concentrations (1.0 mM) rapidly form vinculin-containing (adherens) junctions. Formation of vinculin-containing junctions in closely apposed cells is complete within 15 min as monitored by immunofluorescence. Formation of another epithelial junctional complex in keratinocytes, the desmosome, was previously shown by Hennings and Holbrook (1983) to begin within 5 min after elevation of Ca^{++} when monitored by electron microscopy, but desmosomal staining continued to become more intense for up to 2 h when monitored by immunofluorescence (Watt et al., 1984). The adherens junction staining for vinculin is as intense as that of desmosomes, suggesting that vinculin-containing junctions are not a minor component of the intercellular junction and that they complete their assembly long before desmosomes. Others have noted that adherens junctions may form more rapidly than desmosomes (Kartenbeck et al., 1982).

The zonula adherens, which contains vinculin and into which microfilament bundles insert, has been most extensively studied in intestinal epithelium (Farquhar and Palade, 1963; Staehelin, 1974; Geiger, 1983) and has also been examined in cultured cells, including retinal pigment epithelium (Docherty et al., 1984; Opas et al., 1985) and Madin-Darby

plaques are at the cell periphery in adjacent keratinocytes, although junctions have not yet formed (*b*, *arrow*). Cell borders at the edge of the colony exhibit attachment plaques that are unchanged and remain distant from the periphery (*c*, *arrow*). Within 2 h after switching to 1.1 mM Ca^{++}, all contiguous cells have formed vinculin-containing junctions (*d* and *e*). In some colonies (*e*), parallel vinculin-staining structures are visible; these are magnified in *f* and *g*. Bars: (*a–e*) 20 μm; (*f* and *g*) 4 μm.

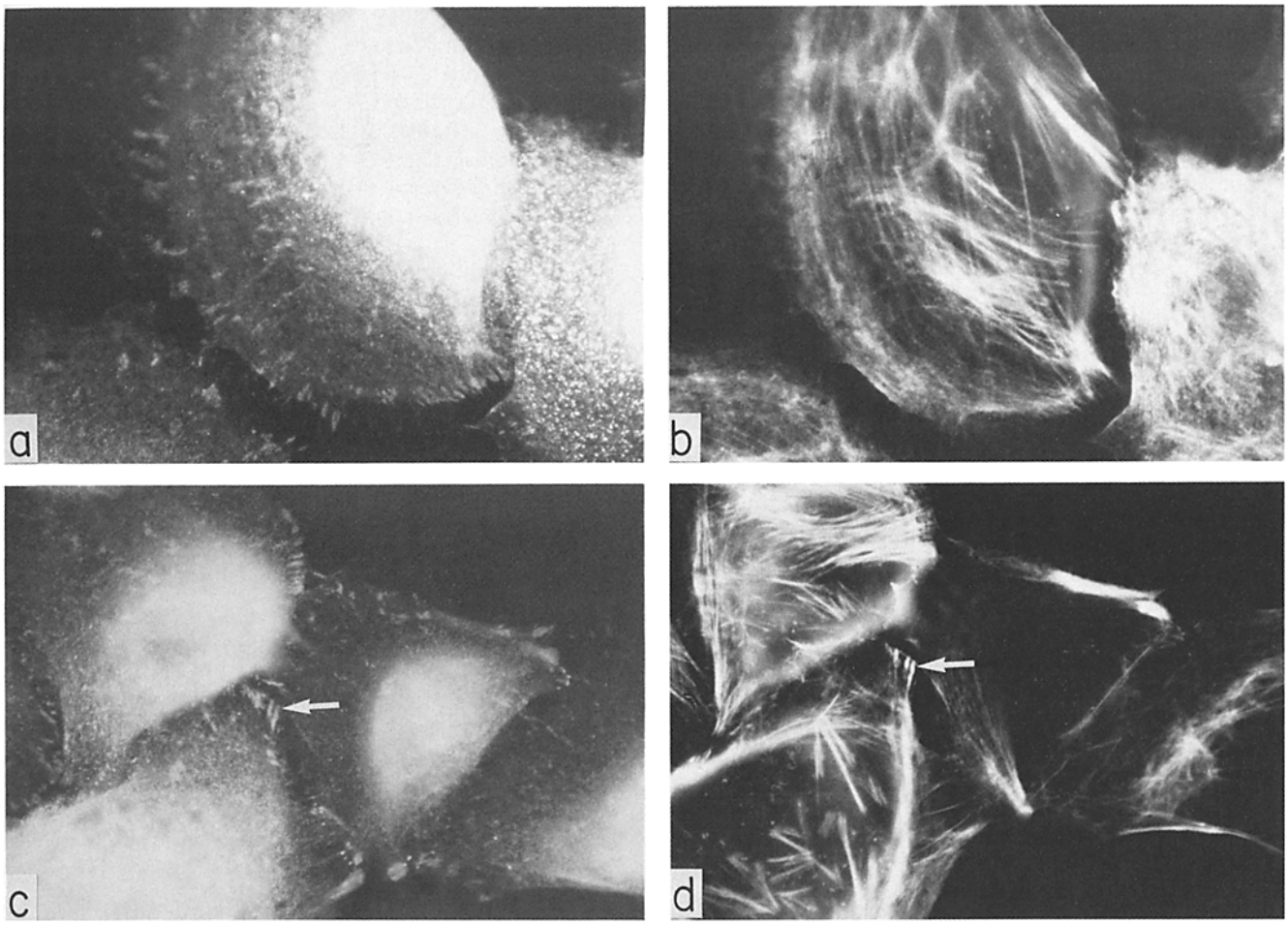
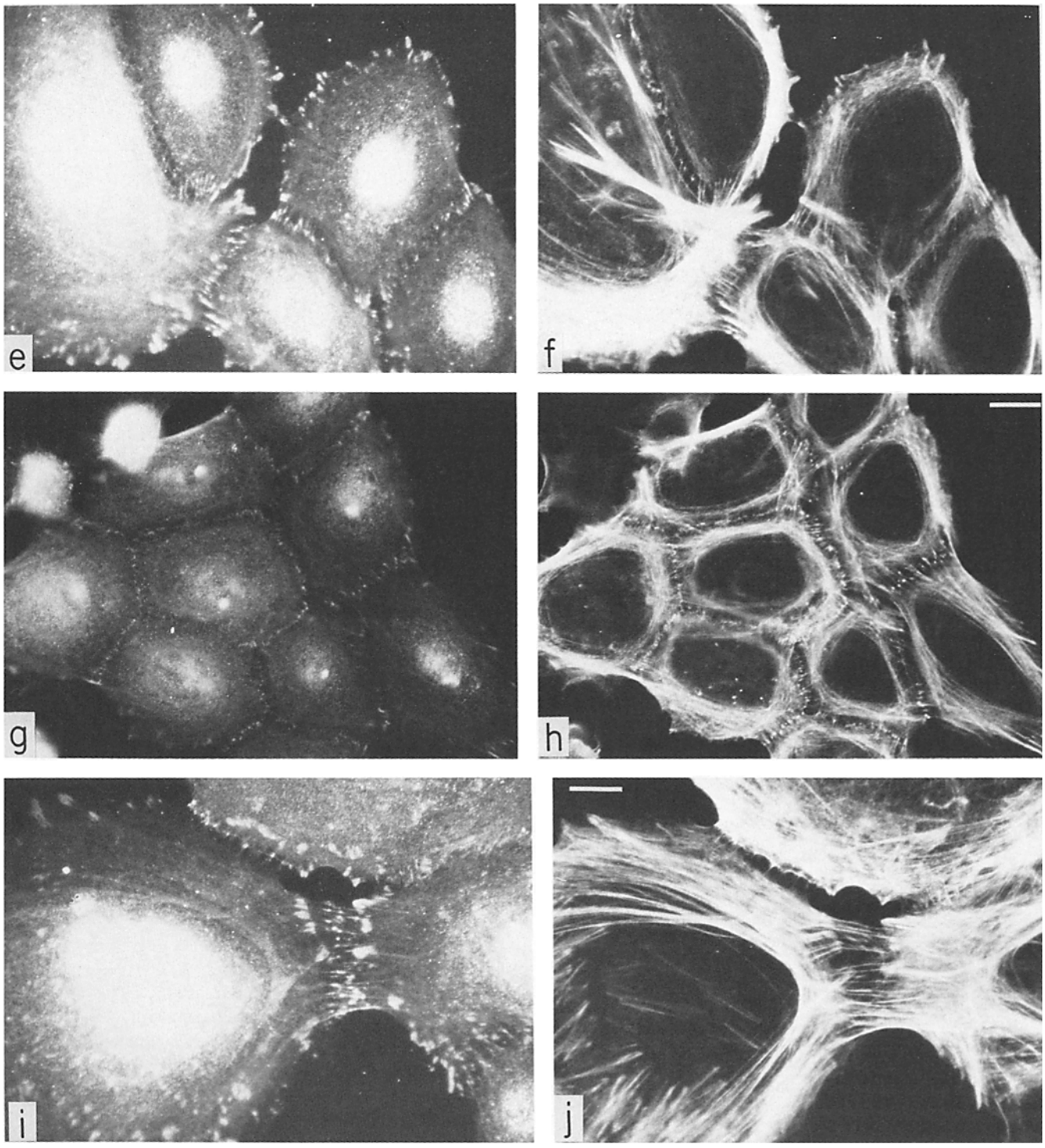


Figure 3. Relationship of vinculin and actin in low and high Ca^{++}. Immunofluorescence of vinculin (*a*, *c*, *e*, *g*, and *i*) and actin (*b*, *d*, *f*, *h*, and *j*). Each pair shows one microscopic field. In cells grown in 0.1 mM Ca^{++}, attachment plaques are at the edge of the cells while actin is in both a diffuse distribution as well as in centrally located stress fibers. Arrows in *c* and *d* indicate coincidence of staining of actin and vinculin in attachment plaque. Within 2 h after switching to 1.0 mM Ca^{++} (*e–j*), vinculin and actin form junctional complexes that appear to connect cells (*e* and *f*) and evolve into fully formed junctions (*g* and *h*). An intermediate stage of junction formation (in adjacent noncontiguous cells after 1 h in 1.0 mM Ca^{++}) shows formation of an actin- and vinculin-containing junction at a time when attachment plaques have not yet been removed (*i* and *j*). Bar, 20 μm.

bovine kidney (MDBK) and mammary epithelial cells (Geiger et al., 1983). Our studies show that keratinocytes rapidly assemble vinculin-containing, actin fiber–associated junctions in proximity to desmosomes.

Electron microscopists have distinguished true desmosomes (macula adherens) associated with tonofilaments (keratin) from small microfilament-associated macular junctions. McNutt and Weinstein (1973) called this latter structure a 70 F-macula adherens to distinguish it from the 100 F-macula adherens, or desmosome. They indicated that the association of actin with the 70 F-macula adherens was a major feature distinguishing desmosomes from these structures (since actin, unlike keratin, is important in cell motility), and suggested a different role for this type of junction. More recently, Geiger et al. (1983) commented on the difficulty of distinguishing these structures by electron microscopy and argued for the use of antibodies to identify them. It is possible to distinguish, based on the type of filaments associated with these different junctions, whether the junction is an adherens type, e.g., a belt desmosome (zonula adherens) or a 70-F macula adherens, both of which contain actin microfilaments (McNutt and Weinstein, 1973; Staehelin, 1974), or, alternatively, a true desmosome (macula adherens) associated with keratin intermediate filaments (Staehelin, 1974). We believe these junctions in keratinocytes should be called adherens junctions because of their associated proteins (actin and vinculin) and because they are distinguishable from desmosomes. Although the distinction between the adherens and desmosomal junctions is possible on the basis of associated filaments, recent evidence for molecular heterogeneity within a given type of junction has been presented (Geiger et al., 1985), and, recently, small plaquelike adherens junctions have been described in cells of intestinal, prostatic, and corneal epithelium (Drenckhahn and Franz, 1986). These may correspond with the 70-F macula adherens of embryonic tissues and cultured cells (McNutt and Weinstein, 1973). Although the nature of the junctional complex requires further study in skin, it is likely that the adherens junctions and desmosomes that form between keratinocytes have an ordered relationship. In intestinal epithelium, for ex-



ample, the desmosome is situated basolaterally in relationship to the more apical adherens junction (Farquhar and Palade, 1963).

Desmosomal structures are not assembled until Ca^{++} is raised. Unlike desmosomal plaques, vinculin-containing structures (attachment plaques) are present at 0.1 mM Ca^{++} in cultured keratinocytes and are a morphologic feature common to both epithelial and fibroblastic cells. Furthermore, junctional adherens-type structures have been noted in both epithelial cells and 3T3 cells (Ungar et al., 1986) and therefore may also be a feature of fibroblast junctions. In 3T3 cells, adherens junctional structures were more developed as cell density increased, and, unlike attachment plaques, were suprabasal in location. Adherens junctions in keratinocytes, as in other epithelial cell types, are located above the ventral surface of the cell (Fig. 4; Geiger et al., 1983; Docherty et al., 1984; Opas et al., 1985). It is of interest that some epithelial and endothelial cells have adherens junctions but have not been found to contain desmosomes by immunologic or electron microscopic studies (Docherty et al., 1984), sug-

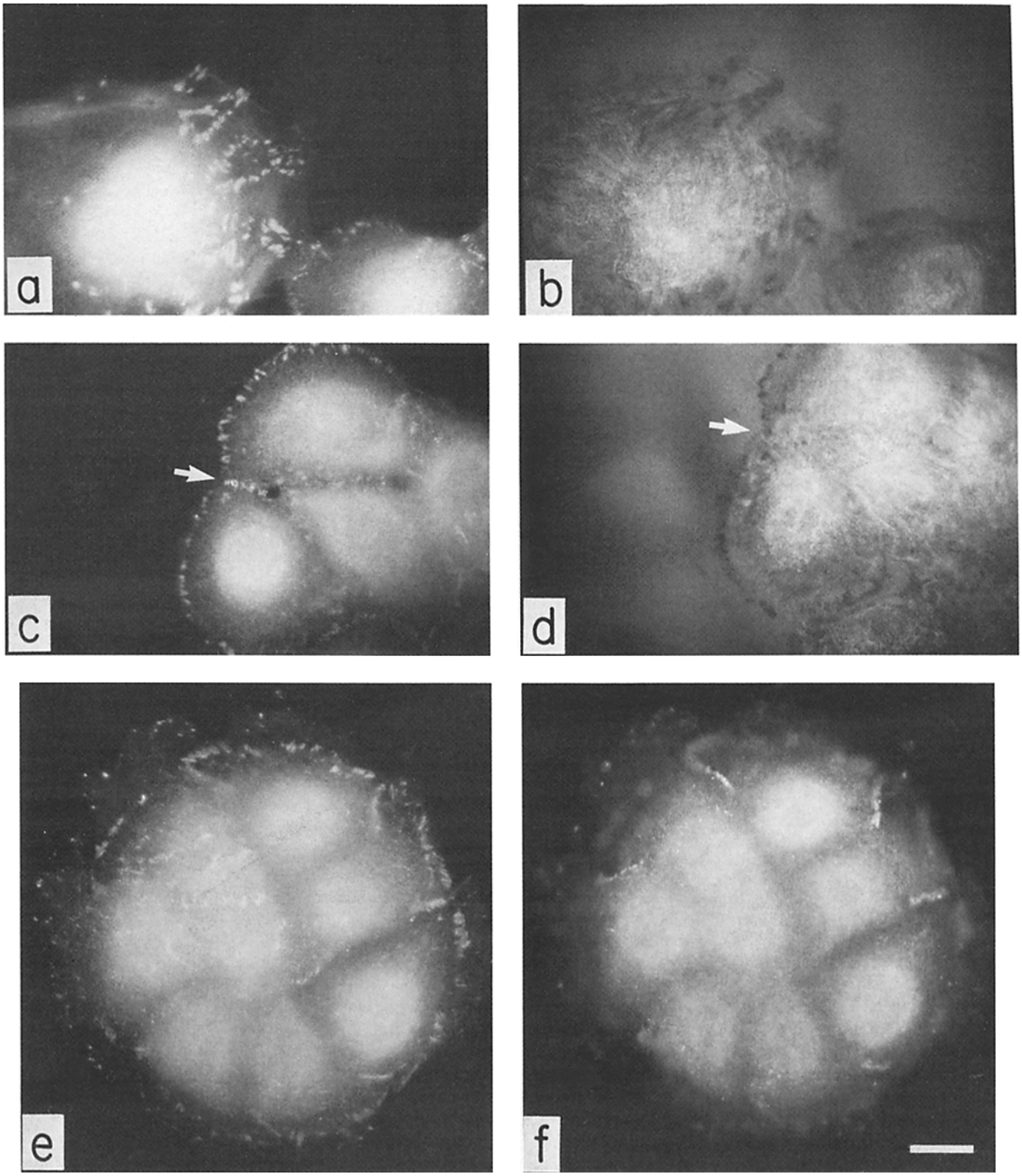
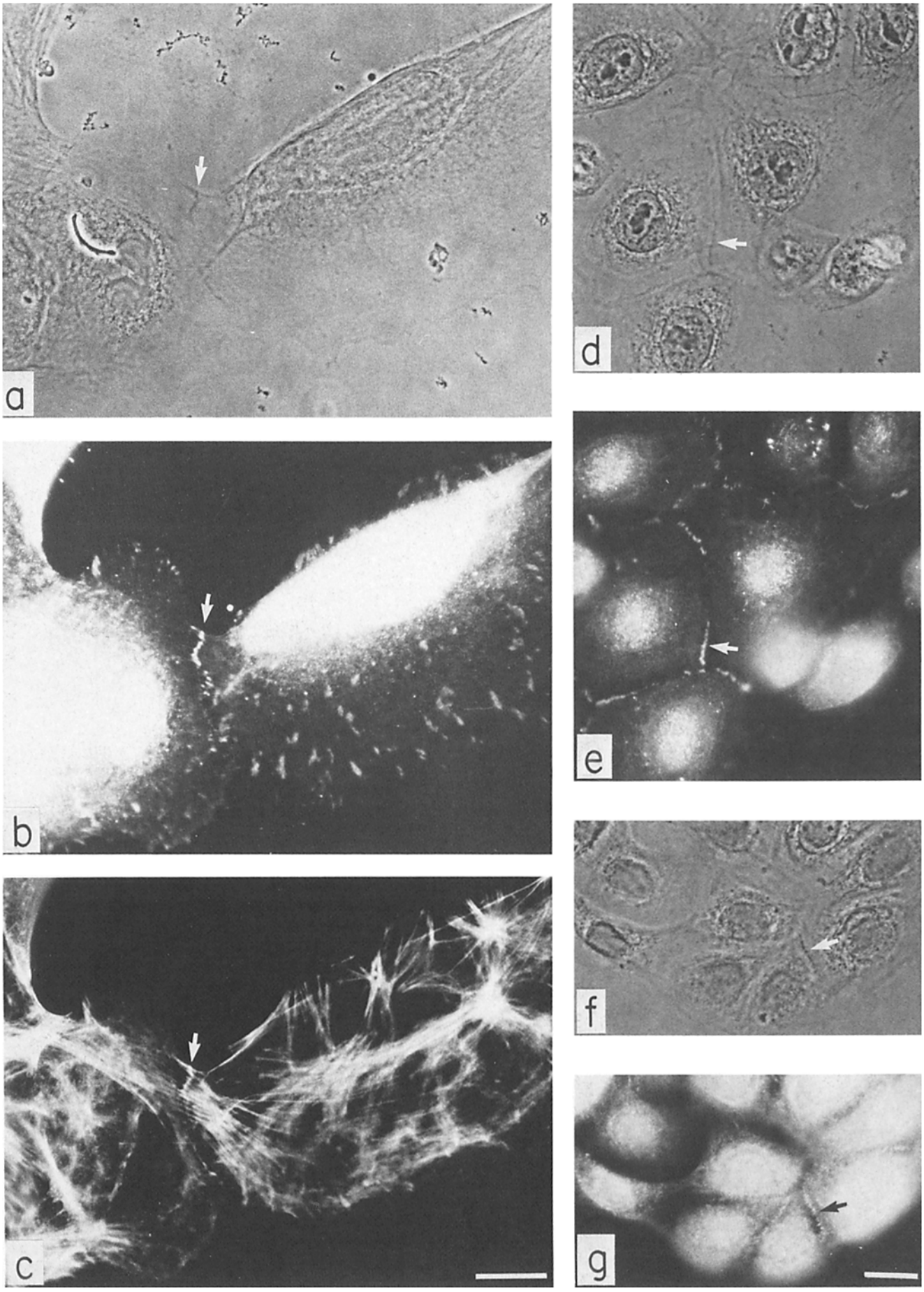


Figure 4. Suprabasal location of adherens junctions shown by interference reflection and by differential focusing. Cells stained for vinculin (*a* and *c*) were examined by interference reflection (*b* and *d*). Cells in 0.1 mM Ca^{++} (*a* and *b*) show correspondence of vinculin staining (*a*) and dark areas on interference reflection (*b*). Cells switched to 1 mM Ca^{++} for 1 h (*c* and *d*) show junctional staining with vinculin (*c*, *arrow*) but the junctional area is not dark in the interference reflection image (*d*). Cells incubated in 1 mM Ca^{++} for 1 h and stained for vinculin (*e* and *f*) show peripheral attachment plaques when viewed at the basal aspect of the cell (*e*), but junctions are better seen at a higher focal plane (*f*). Bar, 20 μm.

Figure 5. Correspondence of adherens junctions with phase-dense structures in keratinocytes. Cells with a single junctional region (*a–c*) or junctions in colonies (*d*, *e*, *f*, and *g*) were examined by phase-contrast (*a*, *d*, and *f*) or by immunofluorescence for vinculin (*b* and *e*), actin (*c*), or desmoplakin (*g*). Actin staining (*c*) corresponds with junctional vinculin staining (*b*) and a dense area in phase-contrast image (*a*) in the same cell. Phase-dense structures may be associated with either vinculin-staining structures (*d* and *e*) or desmosomes (*f* and *g*). Bars: (*a–c*) 10 μm; (*d–g*) 20 μm.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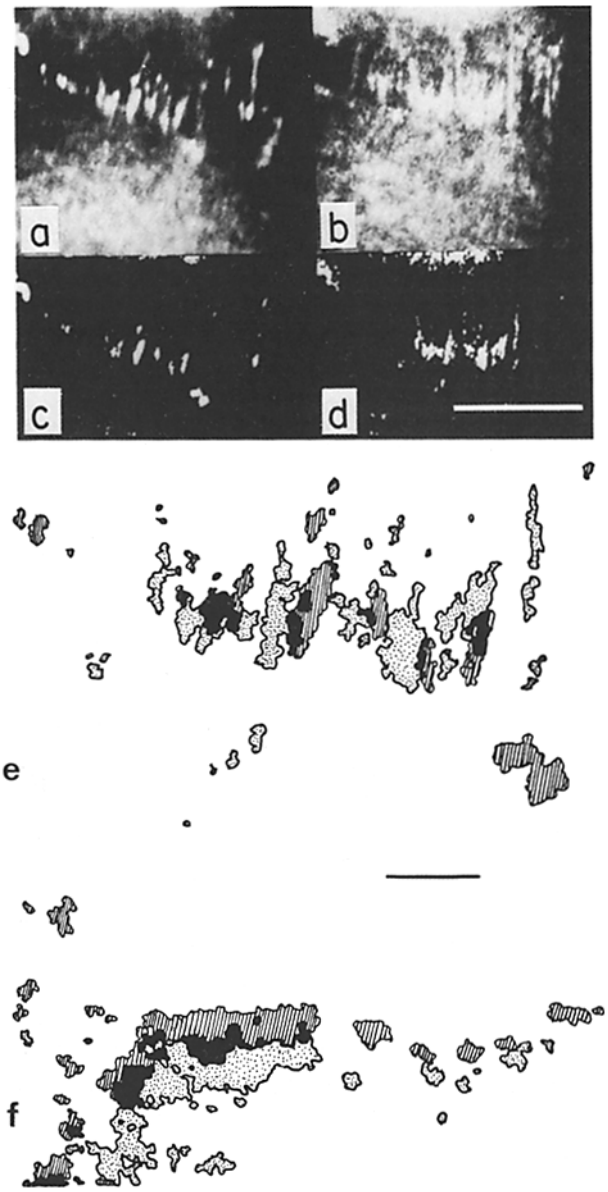

Figure 6. Spatial distinction of adherens junctions from desmosomes in keratinocytes. Video fluorescence images of vinculin-staining structures (*a*) or desmosomes (*b*) from the same intercellular junction were recorded; the images were thresholded to remove nonjunctional fluorescence. The corrected images show the vinculin-staining adherens junctions (*c*) or desmoplakin-staining desmosomes (*d*), and the magnified overlaid images from *c* and *d* are shown in *e*. A second junction is shown in *f*. *Hatched area*, adherens junctions; *stippled area*, desmosomes; *black area*, overlap of adherens junctions and desmosomes. Bars: (*a–d*) 10 μm; (*e–f*) 2 μm.

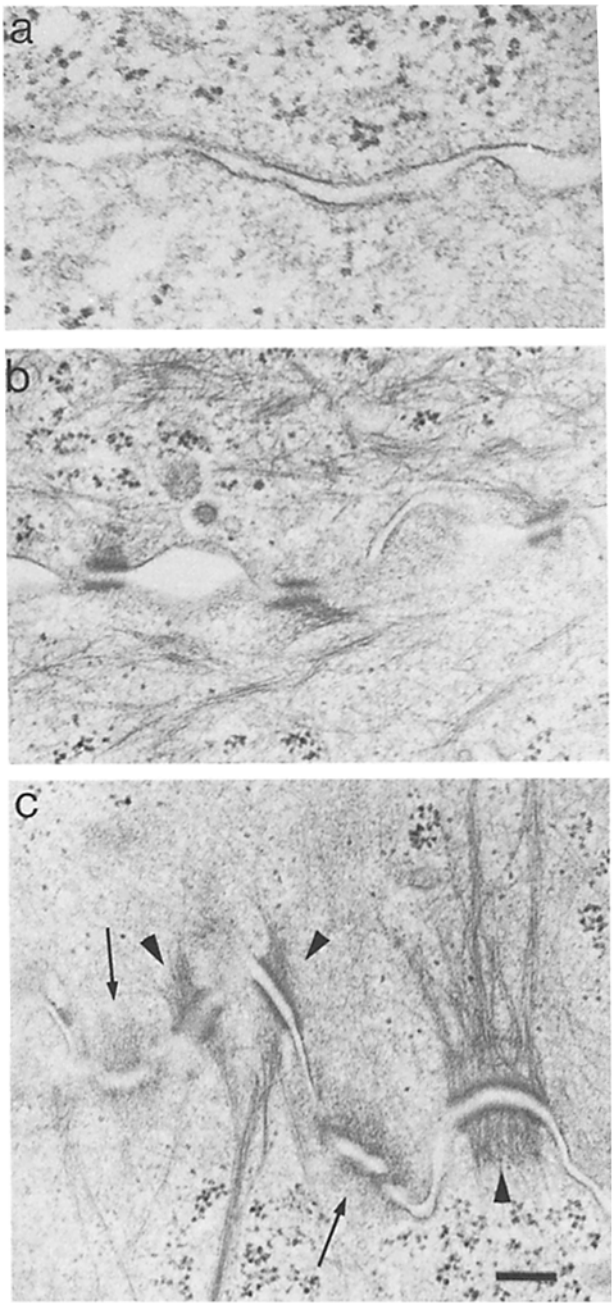

Figure 7. Electron micrographs of junctions formed after Ca^{++} shift. Cells were incubated in medium containing 0.1 or 1 mM Ca^{++} for various times. No junctions were found in control cells in 0.1 mM Ca^{++} (*a*). After incubation in medium containing 1 mM Ca^{++} for 2 h (*b*), adherens junctions are visible. The section shown in *c*, incubated in 1 mM Ca^{++} for 20 h, contains both desmosomes (associated with darkly stained keratin filaments) and adherens junctions (associated with lighter intermediate filaments). *Arrows*, adherens junctions; *arrowheads*, desmosomes. Bar, 0.2 μm.

gesting that adherens junctions may play a substantial role in maintaining structural integrity of the epithelial sheet. In support of this concept, injection of antibody to desmosomes (Cowin et al., 1984) prevented assembly of desmosomes but did not alter morphology of MDBK cells, which are known to contain adherens junctions (Geiger et al., 1983).

At regions of the keratinocyte near junctions that formed in response to elevation of Ca^{++}, attachment plaques disappeared; they were not removed in regions of the cell forming the edge of the colony or in isolated cells. The mechanisms controlling the disposition of vinculin in this manner are obscure. Increasing extracellular Ca^{++} may lead to an increase in cytosolic Ca^{++}; this could activate phosphorylation cascades and may play a role in regulating the deposition of vinculin (Sefton and Hunter, 1981). Alternatively, Ca^{++} influx into cells may activate a Ca^{++}-sensitive protease that would

result in limited proteolysis of vinculin (Herman et al., 1986), leading to the selective removal of vinculin from focal contacts. Last, we do not know whether vinculin deposited in adherens junctions is derived from that in attachment plaques or from an intracellular pool of free vinculin. Substantial amounts of soluble vinculin are present in cells (Rosenfield et al., 1985) and probably produced diffuse fluorescence in our cells stained for vinculin, especially at high Ca^{++}. In this regard, recent evidence suggests that cell density can regulate the amount of new vinculin synthesis (Ungar et al., 1986). Our data show that vinculin-containing adherens junctions are formed after exposure of keratinocyte cultures to physiologic concentrations of Ca^{++}. Further studies will address the mechanism of this process, the relationship between adherens and desmosomal junction formation, and the role of the adherens junction in stratification of keratinocytes.

We thank Richard E. Payne, Jr. and Cole Harris for excellent technical assistance, Dr. Keith Burridge for guinea pig antivinculin antibody, and Dr. Detlev Drenckhahn for antidesmosomal antibody.

Received for publication 20 October 1986, and in revised form 14 April 1987.

Note Added in Proof: Adherens junctions have recently been demonstrated in cultured mouse keratinocytes (Green, K. J., B. Geiger, J. C. R. Jones, J. C. Talian, and R. D. Goldman, 1987, *J. Cell Biol.*, 104:1389-1402).

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