Mechanical Tension Induces Lateral Movement of Intramembrane Components of the Tight Junction: Studies on Mouse Mammary Cells in Culture

DOROTHY R. PITELKA and BARBARA N. TAGGART
Cancer Research Laboratory and Department of Zoology, University of California, Berkeley, California 94720

ABSTRACT Occluding junctions of mammary epithelial cells in nonproliferating primary culture occasionally display an atypical pattern of intramembrane strands oriented predominantly perpendicular, instead of roughly parallel, to the apical border of the junction. To test whether the orienting influence was a centripetal cytoskeletal tension often observed in epithelial sheets on fixed substrates, we seeded cells at low density; this allows them to spread maximally while forming a barely confluent pavement. The result was a fourfold increase in the percentage of junctions with the strongly aligned, atypical pattern. Closely similar configurations were observed as the earliest detectable effect of chelation of extracellular Ca^{++}, which induced pronounced centripetal contraction of the cell body. Externally imposed tension, applied so as to stretch cells in one direction only, affected the positions of strands in stretched junctions as might be predicted, by flattening their undulations, increasing their alignment parallel to the apical border. Thus mechanical tension alone, whether inherent in the cytoskeleton or imposed on the cell surface by exogenous force, can cause coordinate lateral displacement of macromolecular assemblies within the membranes of both joined cells.

A tight junction is a network of lines along which the outer leaflets of the membranes of two adjoining cells appear to be fused (1, 2). The lines of fusion coincide with a pattern of strands within the joined membranes, visible in freeze-fracture replicas. The strand pattern is the same in both membranes (3, 4), indicating that fusion requires concordant structure within both. In the zonula occludens of lining and transporting epithelia, the network of tight-junction strands is a belt that circumscribes the apical surface of each lumen-facing cell and, by binding it continuously to each of its neighbors, restricts transepithelial diffusion through intercellular space (1, 5–7).

Maintenance of the integrity of the zonula occludens thus is essential to normal epithelial function. However, different epithelia—and in some cases the same epithelium in different functional states—characteristically allow different amounts of controlled paracellular leakage of ions and small molecules (6, 7). It follows that the zonula occludens of each epithelial tissue must be so constructed and regulated as to provide the specific degree of tightness or leakiness appropriate to its position and function under prevailing physiological conditions. The average number and configuration of strands within the tight-junction network also show characteristic differences in epithelia of different organs (8, 9) and often in different cell types within a single epithelium (10–12). Little is known, however, of how variations in junction structure are related to relative permeability (9, 13–16).

Evidence from several sources suggests that the junction networks of epithelia normally subject to periodic stretching and relaxation usually are composed of undulating strands, with elongate or rounded meshes (17–19), in contrast to a rectilinear pattern common in other epithelia. The presumption is that stretching of the zonula occludens without loss of continuity is accommodated by flattening of undulations in its strands. Such an adaptation to imposed mechanical stress would require coordinate displacement of the paired strands within the membranes of both cells involved in each affected junction. Mechanisms for cellular regulation of strand positioning and movement are unknown. Current evidence points to the involvement of cortical microfilaments (20–24) but does not clarify the nature of their role.

In the course of experiments designed for other purposes (25) we have observed frequent, uncharacteristic strand patterns in occluding junctions of mammary epithelial cells in primary culture. The atypical arrangement suggested the influ-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 MARCH 1983 606-612

ence of an orienting force acting within the plane of the junction and perpendicular to its axis. We present in this paper evidence that strong atypical alignment of intramembrane strands can be induced by mechanical tension, either inherent in untreated cells or exerted by exogenous agents.

MATERIALS AND METHODS

Cell Culture: Mammary glands of young BALB/c mice, 14–18 d pregnant, were minced; dissociated by incubation in Hanks' balanced salt solution, pH 7.2, containing 1 mg/ml collagenase (Sigma Type I, 210 units/mg; Sigma Chemical Co., St. Louis, MO) and 40 mg/ml bovine serum albumin, for 1–1.5 h at 37°C with agitation; and filtered through 50-μm Nitex mesh (TETKO, Inc., Elmsford NY). Cells were washed and the proportion of epithelial cells was increased by three centrifugations at 900 rpm for 4 min each in culture medium. Viable cells, as estimated by trypan blue exclusion, were counted in a hemocytometer and seeded on plastic petri dishes or multiwell plates at various densities from 3–8 × 10^5 cells/cm^2. They were cultivated in Waymouth's MB 752/1 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 10% calf serum, 10 μg/ml insulin, 5 μg/ml hydrocortisone, and 50 μg/ml gentamycin sulfate.

Under these conditions, the cells undergo one to two rounds of DNA synthesis at most; they form a confluent pavement in 2–4 d. In cultures seeded on plastic at densities of 5 × 10^5 cells/cm^2 or higher, domes (hemicysts) begin to appear at 4–5 d. Their presence is proof of the presence and integrity of zonulae occludentes (6, 26–28).

Cell Stretch Experiment: 20-mm squares of stainless-steel mesh were placed in 35-mm plastic petri dishes; 2 ml of cold, neutralized collagen solution prepared as described elsewhere (25, 29) was poured into each dish to completely cover the screen and allowed to gel. Dissociated cells were seeded on the gel at a density of 3 × 10^5 cells/cm^2 and cultivated as above. Since about twice as many epithelial cells are incorporated in the confluent pavement formed from typical seedings at the same density on collagen as on plastic (30), this constitutes a culture of moderate, not low, density. On day 8 after plating, the substrate was forcibly distorted as follows: self-closing spring forceps were opened maximally and the tips inserted into peripheral meshes of the screen at points equidistant from one of its corners. Gradual closure of the forceps compressed the mesh along one diagonal and increased the length of the other diagonal by ~⅓. With the forceps in place, the culture was incubated at 37°C for 1 h before fixation. Controls were cells seeded at the same density on collagen gels without the screen.

EGTA Treatment: Cell cultures on plastic were treated for 3–50 min with 0.6 μM-0.5 mM ethylene-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) in HEPES-polyvinylpyrrolidone buffer as described elsewhere (25).

Preparation for Electron Microscopy: All cultures were fixed in situ for 30–60 min at room temperature in 1% paraformaldehyde, 3% glutaraldehyde in 0.075 M sodium cacodylate, pH 7.2–7.4. For freeze-fracture, fixed cell sheets in 0.15 M cacodylate buffer were scraped from plastic substrates or stripped with a thin underlying layer of collagen from the gel substrate and transferred to 20% glycerol in cacodylate buffer for 1–4 h. They were frozen in Freon cooled in liquid nitrogen and fractured at −115°C. Cultures for scanning electron microscopy were postfixed in 1% OsO_4 in cacodylate buffer for 30–45 min at room temperature, dehydrated in ethanol, and CO_2-critical-point dried. Dried samples were sputter-coated with ~200 A of Au-Pd. Specimens for thin sectioning, after postfixation in OsO_4, were washed and refrigerated in the dark in 0.5% uranyl acetate in acetate-veronal buffer at pH 6.1, dehydrated through ethanol and anhydrous propylene oxide, and embedded in Epon 812.

RESULTS

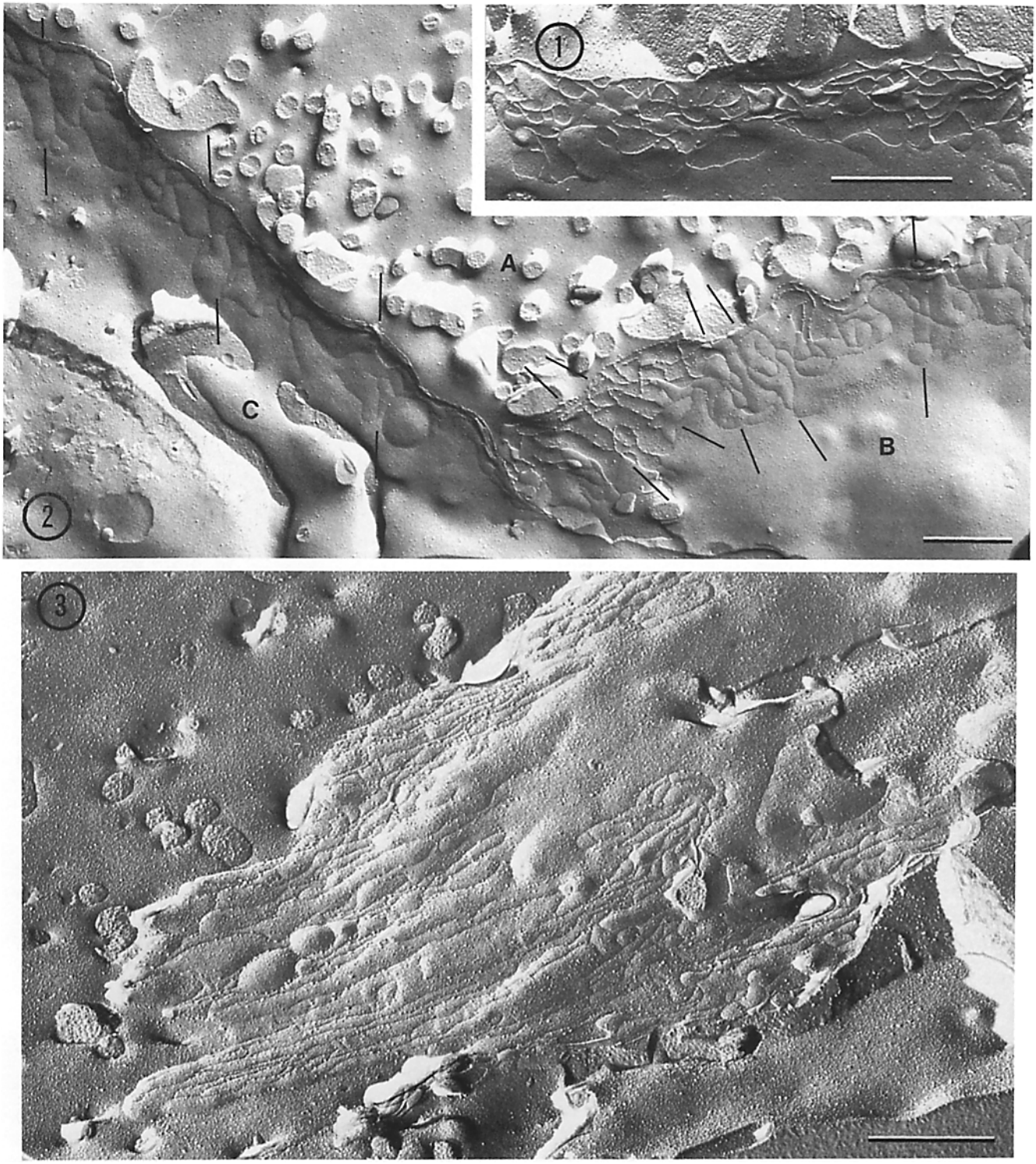
Freshly dissociated mammary epithelial cells seeded at high density on glass or plastic substrates form a typical epithelial pavement of flat, polygonal cells whose upper surfaces, facing the medium, bear microvilli and are connected to neighboring cells by circumferential tight-junction belts. Below the flattened apical surface, cell shape and topography are highly variable. Extensive peripheral overlapping is much more common in these cultures than in intact tissue or in cultures on collagen gel. Where overlap occurs, junctional membranes usually lie in the same plane as the adjacent apical and lateral membranes of the overlapped cell and are approximately parallel to the apical surface of the overlapping cell. As described elsewhere (26), the pattern of intramembrane strands of tight junctions of mammary cells in primary culture generally resembles that typical of nonlactating normal mammary gland in situ. Strands are sinuous, but their predominant orientation usually is roughly parallel to the apical edge of the junction belt, which itself undulates gently (Fig. 1), and their intersections outline meshes with long axes also generally paralleling the apical border. Numbers of strands may vary widely within a culture and even within a single junction.

A subtly atypical pattern occasionally encountered in these cultures is illustrated in Fig. 2, where the intersection of three cells is seen. In the junction between cells *A* and *B*, the continuous apical border strand undulates moderately; predominant orientation of other strands is nearly perpendicular, instead of parallel, to it, and most meshes in the network are elongated in the perpendicular direction. The junction between cells *A* and *C*, seen alone, is not atypical. However, below two or three compactly parallel apical strands, many of the strands and elongated meshes form an angle of 35–40° to the apical border, approaching the orientation in the neighboring *AB* junction. The picture suggests the existence of an orienting force acting on junction *AB* and less strongly on junction *AC*.

That these patterns were not just random variations was suggested by observations made in the course of a separate investigation of the effects of calcium chelating agents on cell and junction morphology (25). Exposure of standard high-density cultures on plastic substrates to buffered solutions of 0.6 μM–0.5 mM EGTA induced centripetal cytoplasmic contraction of the cells, which were still fixed in position by attachment to the substrate and by continuous tight junctions with each other. In cells early in this process, junctions were structurally continuous, but the constituent strands of a high proportion of them were strongly aligned in patterns reminiscent of those in Fig. 2. Fig. 3 represents the junction between one cell and an overlapping extension, ~2 μm broad, of another. Virtually all junction strands are aligned with the long axis of the overlapping process, regardless of the irregular contour of the actual border, and interconnections are infrequent in some areas. The aggregate length of strands appears exceptionally great in proportion to the length of the border. In Fig. 4, the overlapping lip of one cell has been fractured away, exposing the underlying cell's plasma membrane in the tight junction and adjacent apical surface. The apical border of the junction, marked by one continuous strand, undulates strongly. Alignment of most other junction strands nearly perpendicular to the overall direction of the border is conspicuous. Interconnections among strands are reduced.

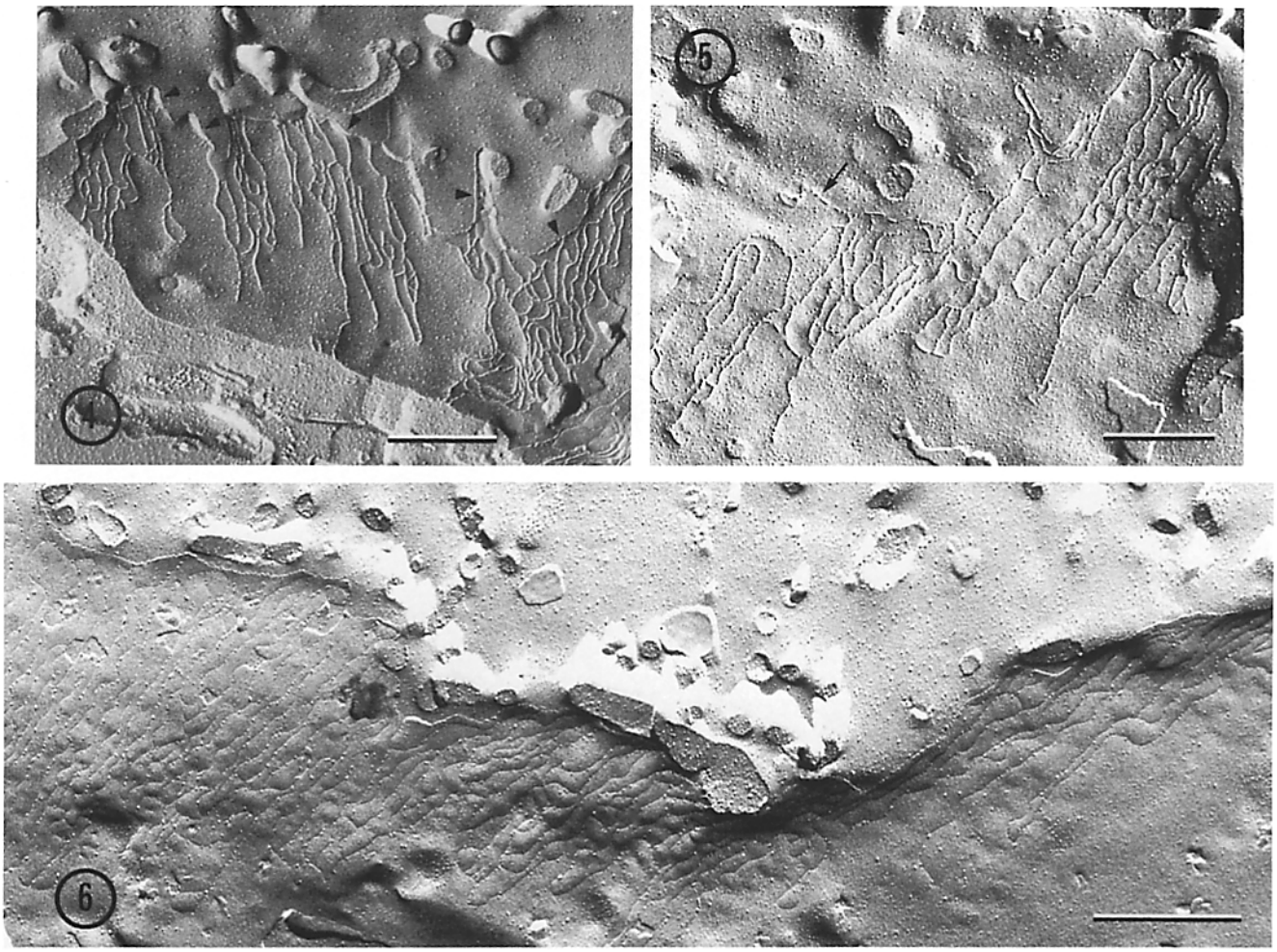
The apparent repositioning of strands illustrated in these two micrographs preceded any other visible effects of EGTA on the junctions. We reasoned, therefore, that it might be caused in part by tension on the peripheral membranes of joined cells resulting from centripetal contraction in one or both, and we determined to examine the effects of mechanical tension in the absence of EGTA or other chemical agents.

Mammary (26, 31) and other (32, 33) epithelial cell sheets spread on a fixed substrate undergo spontaneous contraction if released from the substrate, suggesting that spread epithelial cells are chronically under tension. If so, increased cell spreading might be expected to increase the tension. We therefore seeded mammary cells on plastic at the lowest density that will yield a nearly confluent pavement, ~3 × 10^5 cells/cm^2, and compared tight-junction configuration in these and in cultures seeded at 8 × 10^5 cells/cm^2 or higher. Micrographs were taken of all junction fracture faces in which the apical contour and basolateral extent of the junction were identifiable. They were



FIGURES 1–3 FIGURE 1 This and all subsequent micrographs, except Figs. 7 and 8, are of freeze-fracture replicas. This one illustrates the intramembrane strand network of a typical mammary tight junction, from a culture on collagen gel. The predominant orientation of most strands parallels that of the junction belt itself. Bar, 0.5 μm. × 40,000. FIGURE 2 Intersection of three cells in a high-density culture on plastic. The apical surface of cell A, studded with the bases of fractured microvilli, is bounded by its junctions with overlapping cell B at the right and cell C at the left. The alignment of strands in junction AB is distinctly atypical (compare with Fig. 1) and that in junction AC possibly so. Lines are drawn as extensions of the major axes of meshes outlined by these strands. Bar, 0.5 μm. × 30,000. FIGURE 3 High-density culture on plastic, exposed to 0.5 μM EGTA for 14 min. Part of a cell's apical surface and its junction with a V-shaped overlapping extension of another cell. Elongate, relatively straight strands, aligned parallel to the major axis of the extension, are abundant. Bar, 0.5 μm. × 43,000.

readily separable into two classes, as strand orientation in the atypical junction segments that we consider to be affected by tension is conspicuously more uniform than in typical junctions. Of 36 junction fracture faces from low-density culture, 30 (83%) were clearly of the atypical class. Of 49 junction fracture faces in high-density culture, 11 (22%) were atypical.



FIGURES 4–6 FIGURE 4 Culture and treatment as in Fig. 3. Part of a junction and the adjacent apical plasmalemma, with microvilli, are seen. The apical border strand (arrowheads) is sinuous but continuous. Most strands are conspicuously aligned in a direction nearly perpendicular to the overall course of the junction. Bar, 0.5 μm. × 30,000. FIGURE 5 Low-density culture on plastic. Junction strands are parallel, with few crossbridges; the apical contour of the junction is irregular. A rare exception to the continuity of the apical border strand is seen at the arrow, where one strand extends for a short distance on the apical surface. Bar, 0.5 μm. × 30,000. FIGURE 6 Low-density culture on plastic. An unusual concentration of junction strands, with few cross-bridges, is shown. Although the junction shifts its course about 30°, virtually all of the elongate strands are aligned in a constant direction. Bar, 0.5 μm. × 40,000.

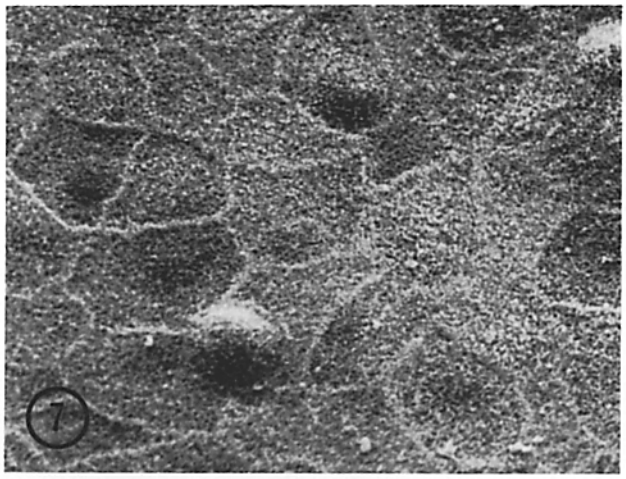
Fig. 5 is an example in which the number and aggregate length of strands appear essentially normal, but alignment approximately perpendicular to the overall course of the junction is pronounced, and cross-bridges between strands are infrequent. In Fig. 6, an accumulation of strands is evident, and, although the apical border itself changes course, all strands are aligned in a common direction.

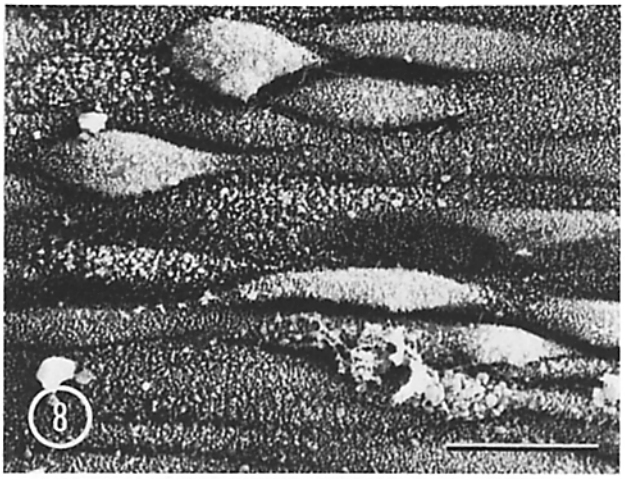
Mammary epithelial cells cultivated on a collagen gel substrate (Fig. 7) never spread as broadly as do those plated on plastic or glass, but retain a diameter and shape more comparable to mammary cells in vivo. To see whether imposed mechanical tension would affect junction pattern under these more natural conditions, we grew cells on layers of collagen gel in which were embedded squares of stainless-steel wire screen and then forcibly distorted these by compressing the screen on the bias. If the gel became distorted with, rather than slipping over, the mesh embedded within it, the expected effect would be stretching of the cells along one axis and compression of the other axis in the plane of the cell sheet. Scanning electron microscopy revealed many areas where the cells had indeed assumed parallel spindle shapes (Fig. 8), interspersed with areas where no shape modification was evident.

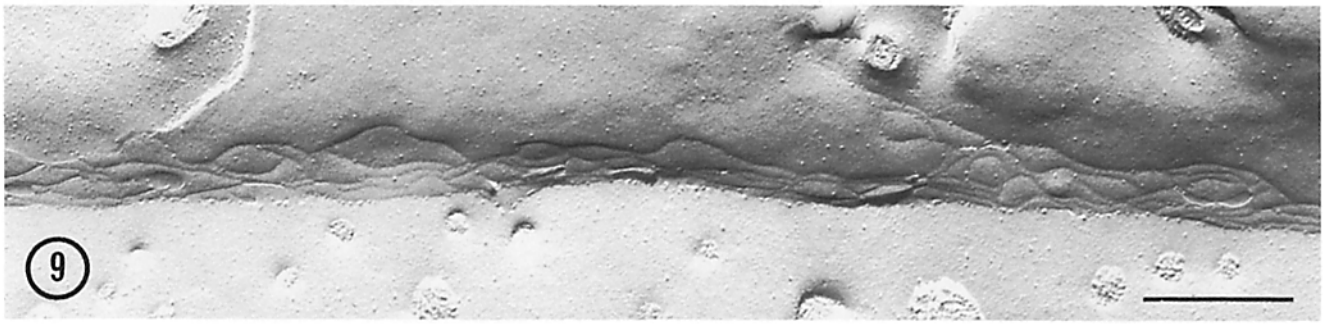
The most striking effect found in freeze-fracture replicas of these preparations was the frequent appearance of extraordinarily straight, long junctions in which the typical orientation of strands generally parallel to the apical border was exaggerated and compacted (Fig. 9). This configuration suggests that a flattening of the normal mild undulations in the apical border strand and reduction in the amplitude of undulation in other strands occurred during longitudinal stretching of the joined membranes of the two cells.

DISCUSSION

We have examined the effects of two different kinds of mechanical tension on tight junctions in nonproliferating primary cultures of mammary epithelial cells. The first kind results from an inherent tonus in the cytoskeleton of epithelial cells in a confluent sheet (32–34). The tight junction conformations that we recognize as typical in mammary cells are compatible with this tonus in high-density cultures. However, when cells are seeded on plastic at a density at which they must spread







FIGURES 7–9 FIGURE 7 Scanning electron micrograph of a moderate-density control monolayer on collagen gel. The cells' apical surfaces, outlined by hedgerows of close-set border microvilli, are polygonal and flat or gently rounded. Bar, 10 μm. × 1,500. FIGURE 8 Scanning electron micrograph of cells at moderate density on a collagen gel, fixed 30 min after application of tension by distortion of a wire screen embedded in the gel. The cells have been stretched in one direction and compressed in the other. Compression causes bulging of apical surfaces, and hedge rows of border microvilli, if present, are hidden in the grooves. Bar, 10 μm. × 2,000. FIGURE 9 Junction from a culture exposed for 55 min to mechanical tension as in Fig. 8. As compared with the junction between control cells on gel in Fig. 1, the amplitude of undulation of both the border and other strands is reduced, the average mesh shape is flatter, and the depth of the junction, relative to the number of strands in it, is decreased. Bar, 0.5 μm. × 40,000.

broadly in order to cover the substrate, cytoskeletal tension apparently is increased. Under these circumstances, the same tonic centripetal forces that gradually contract mammary cells and their supporting collagen lattice in a floating gel culture (31) will exert tension on the peripheral plasma membranes. These membranes cannot retract, since they are bound by continuous zonulae occludentes to neighboring cells within which similar centripetal tension exists, and most cells in the pavement are also attached to the substrate. The paired membranes of any given junction thus will be subject to the opposing tonic forces of the two cells involved. Where one cell is overlapped by another, their mutual junction belt, lying in a plane parallel to the flattened cell surfaces, will be pulled in opposite directions at its apical and basal borders. We suggest that these sustained tensions are responsible for alignment of tight-junction intramembrane strands generally parallel to the direction of the locally predominant force. Local variations in cell density, and changes over time in the shapes and cytoskeletal configurations of individual cells or in the extent and topography of cell overlap, could all modify the degree and angular direction of tension on any junction segment. Such local variations in high-density cultures may explain the fraction (~20%) of junctions showing atypical orientation.

Withdrawal of extracellular calcium, in addition to causing a strong centripetal contraction of the cell body, destabilizes the tight junction strands, increasing their lateral mobility (35, 36). In our EGTA-treated mammary cells, tension on junctional membranes during the first moments of contraction caused a rapid repositioning of paired intramembrane strands in high-density cultures, mimicking and exaggerating the effect of sustained tonus in untreated low-density cultures.

The second kind of mechanical tension we investigated was externally applied to cells cultivated on collagen gel, where cell-cell relationships more nearly resemble those in intact nonlactating tissue. By distorting the substrate, we stretched the cells in one dimension only. Here we found none of the strand configurations we attribute to centripetal intracellular tension in a confluent pavement. Instead, stretched junctions were exceptionally straight and narrow; the effect was like that of tension on a loosely braided cable. This is the kind of effect predicted for similarly patterned junctions in normal epithelia stretched in vivo (17–19).

Both of these tensions—one internally generated and pulling radially on the circumferential junction belt, and the other imposed by distortion of the substrate, stretching the junction belt along its axis—influenced the alignment of tight-junction strands. The first kind of tension results in strand patterns, to our knowledge not previously reported. We have no evidence as to whether it affects junction permeability. If only the single apical border strand persists as a continuous line of occlusion, it could in theory provide an effective permeability barrier. Low-density cultures do not form domes (37), but this could be related to extent of cell spreading and substrate attachment or to low transport activity as well as to junction properties.

The second type of tension causes a modest displacement of a kind that, in vivo, probably helps to maintain junction tightness during predominantly unidirectonal periodic stretching.

Several authors have examined effects of prolonged intraluminal hydrostatic pressure on tight-junction configuration in liver (38, 39), pancreatic (40), and epididymal (41) epithelia in vivo after duct ligation. The expected effect of increased intraluminal pressure on epithelial lining cells would be general flattening with accompanying forced increase in cell diameter and circumference. Strand order and interconnections became disorganized in distended liver and pancreas, with or without the appearance of discontinuities in the junctions. In the epididymis, ligation induced an increase in numbers of strands and in their convolutions, often in close parallel formation. Lateral displacement of strands occurred in all cases cited, but no vectorial influence was evident. None of these patterns has resembled those generated by tension in our experiments.

The aggregate length of strands in our stressed junctions is such as to indicate that at least all of the typical complement is present and assembly of new strand lengths often may have occurred. Lateral migration and unpatterned proliferation of tight-junction strands are recognized as a common epithelial response to a variety of other experimental conditions (42) and so might be expected here. The peculiarly orderly alignment of strands in our material, however, illuminates aspects of tight-junction organization not noted before.

The apical border strand of the tight junction defines the linear border between the apical surfaces of the two cells. All strands of the typical, normal junctional network are interconnected. Under the influence of centripetal tension, anastomoses and cross-bridges among subapical strands are progressively reduced. However, they retain their insertions on the apical strand and in effect hang from it, singly or in linked groups, like clothes from a clothesline. The apical strand is capable of lateral displacement and often becomes strongly undulatory under tension, carrying with it the insertions of the other strands.

It is not unreasonable to assume that tight-junction intramembrane strands are normally connected somehow to a cortical microfilament network or can acquire such connections under the influence of tension. If this is so, centripetal contractile tension involving radiating actin cables might entrain microfilaments of the cortical meshwork, creating centripetal axes of tension in it, transmissible to macromolecular assemblies in the membrane. Comparable stresses would probably occur in both cells involved in a junction, but they need not be equal or precisely opposite. Conflicting tensions could distort the common cell border and the intramembrane apical strand that marks it. Our evidence suggests that the majority of other junction strands are repositioned along lines parallel, and therefore offering least resistance, to the locally prevailing stress vector, while remaining anchored on the apical border strand. The border strand thus appears to have a critical role in the maintenance of the occluding junction as a unit.

We are grateful to the Electron Microscope Laboratory, University of California, Berkeley, for the use of its freeze-fracture and scanning electron microscopy facilities, to John Underhill and Lionel Thomas for assistance with photographic procedures, and to Susie Castillo for expert secretarial work.

Our work was supported by National Institutes of Health grant GM26230, Department of Health and Human Services.

Received for publication 17 May 1982, and in revised form 3 November 1982.

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