

In Vitro, Rapid Assembly of Gap Junctions Is Induced by Cytoskeleton Disruptors

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ABSTRACT We report here rapid assembly of gap junctions in prostate epithelial cells in vitro. Assembly of gap junctions can be induced by incubation at 0°C followed by incubation at 37°C. Colchicine (10^{-5} M, 10^{-3} M) and cytochalasin B (25 μ g/ml, 100 μ g/ml) at room temperature or at 37°C also induce assembly of gap junctions. Assembly of the junctions proceeds even in the presence of a metabolic inhibitor (dinitrophenol) or of an inhibitor of protein synthesis (cycloheximide). We conclude that assembly of gap junctions can proceed from a pool of pre-existing precursors. The experimental conditions that result in gap-junction assembly involve perturbation of the cytoskeleton. Therefore, we propose that the assembly of gap junctions requires convergent migration of precursor molecules whose positional control in the membrane is released by perturbation of the cytoskeleton. Aggregates of particles and rugosities, whose distribution size and shape is similar to that of gap junctions, may represent intermediate assembly stages. This would indicate that the final stages in the assembly take place only after convergence of the precursor molecules to the junctional site and involve profound conformational changes required for establishment of fully assembled connexons.

Gap junctions are regional membrane specializations that, in animal cells, are responsible for intercellular communication. Biochemically, protein components of gap junctions have been isolated and characterized (see references 2, 16, 23, 33 for reviews). Electron microscopic and spectroscopic studies indicate that the junctional proteins are arranged into hexamers that form "connexons," the structural and functional units of the gap junctions. In each connexon, the hexamers form a rosette that delimits a central hydrophilic channel (4, 12, 35).

The mode of formation of gap junctions is obscure and the forces responsible for their structural maintenance are largely unknown. Freeze-fracture studies indicate that the gap junctions are formed by convergence, through translational migration of large intramembrane particles (1, 7, 19). The junctions appear to be highly dynamic structures whose frequency, size, and pattern of distribution respond to a variety of physiological stimuli (3, 6, 8, 11, 17, 18, 32, 36). Structural changes in gap junctions, observed as differences in packing of connexons, also occur in response to conditions that cause electrophysiological uncoupling (22, 24, 25, 26). At present we know little about the molecular events that lead to formation of the connexons. In particular, it is not clear whether the connexons are (or originate from) pre-existing integral membrane proteins or whether the gap junctional proteins must be synthesized *de*

novo. A major obstacle to these investigations is the lack of experimental models in which formation of gap junctions *de novo* can be induced under controlled conditions. At present, there are several *in vivo* approaches to study the formation of gap junctions, but they require long incubation periods (6, 9, 17). In addition, it is difficult or impossible to distinguish the primary (i.e. direct) effects of the stimulus from the secondary changes it generally induces. In tissue culture, cells display few gap junctions and experimental procedures that could induce their proliferation remain unknown (28).

Recently, we showed that the structural elements of another specialized intermembrane contact—the tight junction or "zonula occludens"—can be induced to proliferate in a massive and rapid manner by a simple procedure (15). Incubation of excised rat ventral prostate tissue for 5 min or more at 37°C results in the formation of numerous and long junctional strands over the lateral membranes of columnar epithelial cells. The length of these strands can reach up to 12 times that originally present in the tight-junction band. These conditions, however, do not lead to parallel increase in the frequency of gap junctions which, in these prostate epithelial cells, remains low. We report here methods that, *in vitro*, induce notable increases in the frequency of gap junctions in rat prostate. These methods involve conditions that lead to perturbation of

the cytoskeleton. Assembly of gap junctions occurs within 10 min of the time of excision and is not affected by metabolic or protein synthesis inhibitors.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (150–175 g) were lightly anesthetized with ether and the bilobed prostatic tissue was excised. In each instance, one lobe was used to test the experimental conditions and the other used as a control. Each experiment was repeated three times with tissue taken from different animals. Tissue was minced in 0.1 M sodium cacodylate buffer at pH 7.4 and incubated in the same buffer under the desired experimental schedule. Some experiments involved temperature shifts. A rapid temperature transition was assured by placing the tissue within vials with a wire-mesh bottom that could be quickly transferred to buffers at different temperatures. The tissues were incubated at 0°C for 2, 5, 15, or 30 min and then transferred to 37°C and incubated for an additional 2, 5, 15, or 30 min. Temperature shift experiments (30 min at 0°C; 30 min at 37°C) were also performed in the presence of cycloheximide, a protein synthesis inhibitor (27), or of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation (5, 14). These drugs were added to the incubation medium at both temperatures in concentrations of 200 µg/ml and 5 mM, respectively.

To assess the effect of chemical disruptors of the cytoskeleton, we incubated minced tissue (at room temperature or at 37°C) for 60 min in colchicine (10^{-3} M and 10^{-5} M) or in cytochalasin (100 µg/ml or 25 µg/ml). A shorter incubation period (10 min at room temperature) was also tried on higher concentrations of the drugs (colchicine 10^{-3} M, cytochalasin B 100 µg/ml). To test the reversibility of the effects of colchicine and cytochalasin B, we removed some treated samples after 30 min, washed them and incubated them at 37°C for 30 min in buffer without the drugs. Experiments with cytoskeleton disruptors were also performed with temperature shifts as described above (i.e. preincubation with the drug at 0°C for 30 min followed by incubation at 37°C for 30 min).

To rule out the possible toxic effects of arsenic in cacodylate as well as the effect of the absence of Ca^{++} , Mg^{++} , K^{+} , and Cl^{-} ions from the incubation medium, we repeated all experiments using Tyrode buffer bubbled with a gaseous mixture of 95% oxygen, 5% carbon dioxide (24, 25).

All experiments were terminated by fixation for 2 h in 5% glutaraldehyde in cacodylate buffer at the temperature of the last incubation. The specimens were rinsed in buffer, impregnated in 30% glycerol in buffer, placed on gold specimen carriers, and frozen in partially solidified Freon 22. The frozen specimens were freeze-fractured at -130°C and shadowed at an angle of 45° with a platinum/carbon electron gun. The replicas were cleaned, mounted on Formvar-coated grids, and observed with an electron microscope at 80 kV.

Morphometry: For each experimental condition we photographed and printed (final magnification, $\times 32,500$) at least 25 views of distinct areas of fractured lateral membranes displaying increase in gap junction frequency. The minimum total area of fractured lateral membrane scanned in each experiment was in excess of 2,000 μm^2 . Gap junctions were identified, counted, averaged, and their average frequency was expressed as the number of gap junctions per 100 μm^2 . The maximum density of gap junctions observed on fractured faces of lateral membranes (area $>20 \mu\text{m}^2$) was also recorded.

RESULTS

In the prostate, epithelial cells are prism-shaped with five to seven faces. A well-developed tight-junction band seals the epithelium at the apical pole. Gap junctions are seldom observed. They were detected only over 20–25% of fractured lateral membranes.

Frequency or shape of gap junctions was not altered if, after excision, the tissue was incubated at temperatures ranging from 0° to 37°C. As expected (15), incubation at 37°C for 5 min or more resulted in striking proliferation of tight-junction strands (Fig. 1). Proliferation of tight junction strands also occurred at room temperature after long incubations (30 min or more). However, incubation at 37°C for 2 min or at room temperature for 10 min failed to induce such proliferation except for some loose tight junction strands observed in some lateral membranes.

While incubation at 37°C did not, by itself, induce increased frequency of gap junctions, we found that striking assembly of gap junctions occurred if the tissues were preincubated for 30

min at 0°C followed by incubation for an equal period at 37°C (Figs. 2 and 3; see also Table I). Here the gap junctions appeared distributed along the entire face of lateral membranes, with some smaller areas (generally closer to the apical region) displaying an even higher concentration of junctions. The junctions were frequently small, round or oval, (15–20 connexons) (Fig. 3), but in other instances they could be much larger and of irregular shape (Fig. 2). In contrast to control tissues where typically more than half of the lateral membranes display no gap junctions, a majority of lateral plasma membranes possessed gap junctions. Observation of gap junctions at transitions of the P face of the plasma membrane of one cell to the E face of the apposed cell (see Fig. 5a, arrowheads) showed that the particle aggregates in P faces and arrays of pits over E faces did correspond to intercellular contacts. The frequency of gap junctions was highly variable even within contiguous lateral faces of the same columnar cell, or even within a single lateral face. These variations are reflected in the difference between average and maximum densities of gap junctions (Table I).

We attempted next to shorten the incubation periods necessary for induction of gap junction assembly. We found that increase in the number of junctions occurred even when the tissues were incubated immediately after excision for 5 min at 0°C followed by 5 min at 37°C (Fig. 4). However, incubation for 2 min at 0°C, followed by 2 min at 37°C, failed to induce assembly of gap junctions. The frequency of gap junctions under various experimental schedules is shown in Table I. Gap junctions were occasionally observed within the apical tight-junction band (Fig. 2, arrowhead). The extent of gap junction assembly was unaltered if the tissues were preincubated in DNP (10^{-3} M) or cycloheximide (200 µg/ml) for 30 min at 0°C followed by incubation for 30 min at 37°C (Figs. 2 and 3). This showed that the assembly of gap junctions could occur in the presence of metabolic inhibitors or in the presence of inhibitors of protein synthesis.

The need for a preincubation at 0°C to induce assembly of gap junctions led us to hypothesize that some dissociation of the cytoskeleton was a necessary condition. Clearly, the incubation in cytoskeletal disruptors could not be done at 0°C, as their effect would be superimposed on that of low temperature. Therefore, we incubated freshly excised prostatic tissue in solutions of colchicine (10^{-3} M) and cytochalasin (100 µg/ml) at room temperature or 37°C for 60 min. The results—assembly of gap junctions (and, also, of tight junction strands)—confirmed our expectations (Fig. 5a and b). Similar increases were also observed in tissues treated with the same concentration of colchicine (10^{-3} M) for a shorter period (10 min). Cytochalasin B (100 µg/ml) under similar conditions did not induce apparent increases in the frequency of gap junctions. Lower concentration of colchicine (10^{-5} M for 60 min) induced assembly of gap junctions, but lowering the concentrations of cytochalasin B (25 µg/ml for 60 min) resulted in marked reduction (Table I). Further incubation of the tissue for 30 min at 37°C in the absence of colchicine or cytochalasin B, failed to reverse their effects. Temperature shift experiments performed in the presence of colchicine or cytochalasin did not result in further increase in the number of gap junctions (Table I).

We searched then for membrane modifications that might be related to the process of formation and/or assembly of gap junctions. We found no evidence that the junctions were assembled through a gradual process of accretion of large, rounded particles. In general, even small gap junctions had 10–15

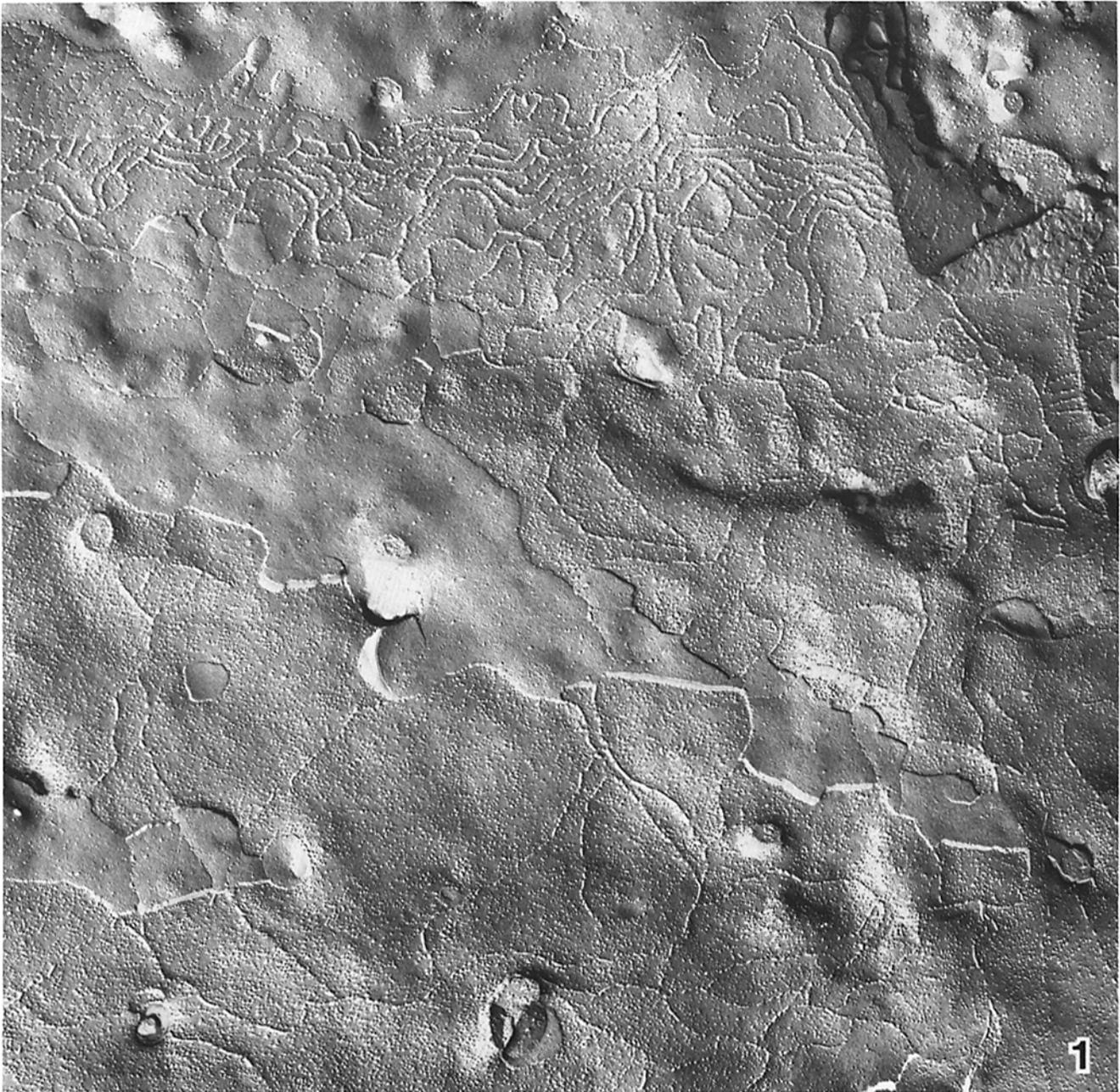


FIGURE 1 Assembly of tight junctions induced by temperature: prostate tissue was incubated at 37°C for 10 min and fixed. Massive assembly of tight-junction strands is not accompanied by an increase in frequency of gap junctions. $\times 36,500$.

connexons or more. In the areas of intense proliferation of gap junctions, we consistently found small and typical clusters of heterogeneous membrane particles with a size, shape, and pattern of distribution similar to that of gap junctions (Fig. 6). They were sometimes found at the tip of tight-junction strands (Fig. 6a, white arrow), a position frequently occupied by gap junctions (Fig. 6c, white arrows). The clusters had a texture similar to that of desmosomes next to the apical tight-junction band. They were, however, smaller (0.08–0.1 μm diameter) and, on P faces they occupied sharply raised areas, whereas the larger desmosomes generally occupied depressed or, less frequently, flat areas (Fig. 2, asterisk). The small clusters of particles existed always in lateral membranes without any additional aggregation of intramembrane particles. In some instances the aggregates were composed of larger particles and

fewer rugosities, always in coexistence with gap junctions (Fig. 6d and e).

DISCUSSION

We show here that it is possible to induce, *in vitro* assembly of gap junctions. The experimental conditions that induced this assembly can be related to the disruption or perturbation of elements of the cytoskeleton. Assembly of gap junctions occurred in the presence of concentrations of dinitrophenol known to inhibit cellular metabolism, or of cycloheximide under conditions that prevent significant synthesis of protein molecules. Therefore, we propose that the assembly of gap junctions can proceed from moieties already present within the plasma membrane at the onset of the experiment.

While tight-junction strands proliferate freely upon short



FIGURES 2 and 3 Assembly of gap junctions induced by temperature: Prostate tissue was incubated at 0°C for 30 min and then transferred to buffer at 37°C for an equal period. Assembly of gap junctions occurs even in the presence of cycloheximide (Figs. 2 and 3) or of DNP. Gap junctions may be large and pleomorphic (Fig. 2) or consist of small, round, or oval aggregates of connexons (Fig. 3). Gap junctions are easier to see over P faces; close inspection of E faces shows characteristic arrays of pits (Fig. 3, arrowheads). Gap junctions are occasionally observed within the apical tight-junction band (Fig. 2, arrowhead). The density of gap junctions in both micrographs is $\sim 166/100 \mu\text{m}^2$ (there are ~ 30 gap junctions in each micrograph). Asterisk: desmosomes at the apical band. Fig. 2, $\times 47,000$; Fig. 3, $\times 35,000$.



incubations at 37°C (more than 2 min), gap junctions do not: they require a preincubation, albeit short, at 0°C. At this temperature, microtubules dissociate (10, 21, 34). Complete depolymerization of the microtubules generally requires periods of incubation at low temperature longer than those used

in our experiments. Long periods of incubation are also required for completion of the cytoskeleton disruption by either colchicine or cytochalasin B (20). Therefore, the short periods (10 min) of incubation required in our experiments for assembly of gap junctions to occur suggest that incomplete depoly-

TABLE I
Frequency of Gap Junctions under various Experimental Conditions

	Incubation periods (min)			Number of gap junctions per 100 μm^2	Maximum observed frequency per 100 μm^2
	0°C	RT	37°C		
Control	—	—	10	1.9	6
	—	—	60		
	—	60	—		
Temperature shift	5		5	28	68
	30		30	29	76
Temperature shift + DNP	30		30	30	68
Temperature shift + cycloheximide	30		30	31	166
Temperature shift + colchicine 10^{-3}	30		30	29	109
Colchicine 10^{-3}	—		60	28	40
	—	60	—	25	70
	—	10	—	25	75
Colchicine 10^{-5}	—	60	—	22	82
Temperature shift + cytochalasin B	30	—	30	23	38
Cytochalasin B 100 $\mu\text{m}/\text{ml}$	—	—	60	20	35
Cytochalasin B 25 $\mu\text{m}/\text{ml}$	—	60	—	17	35
	—	60	—	15	60

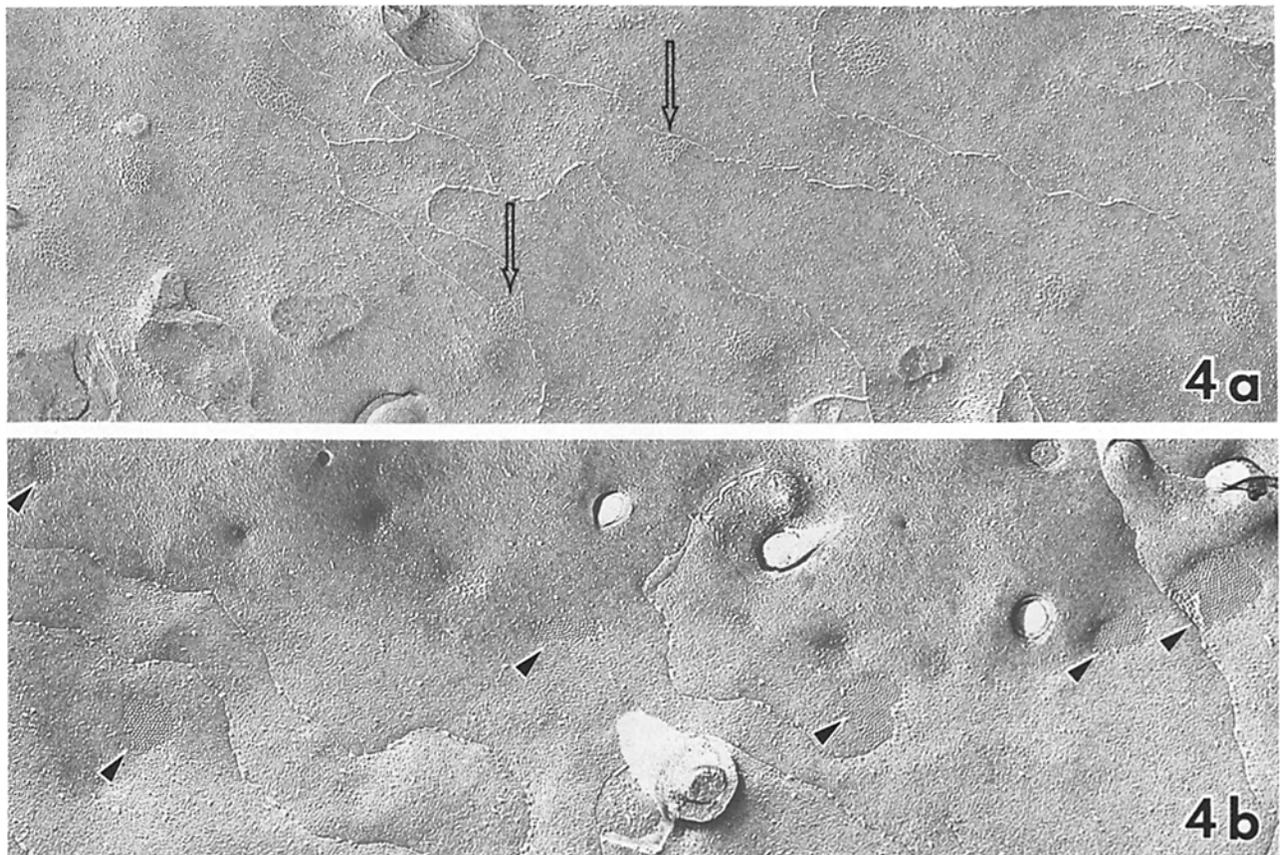


FIGURE 4 Short-term induction of gap-junction assembly. This tissue was fixed in 5% glutaraldehyde 10 min after excision of the prostate: 5 min at 0°C followed by 5 min at 37°C. In *a*, gap junctions can be seen sharing the side of tight-junction strands (arrows). In Fig. *b* the arrays of pits characteristic of the E faces of gap junctions are revealed (arrowheads). *a*, $\times 40,000$; *b*, $\times 50,000$.

erization of some of the cytoskeleton components may suffice. Recent studies show that *in vivo* injection of colchicine, vinblastine, or vincristine causes significant increase in the surface areas of gap junctions (29–31).

We propose that assembly of gap junctions involves the

convergent migration of junctional precursor molecules whose positional control within the membrane is released by perturbation of cytoskeletal elements. This might take the form of a rather specific breakage of molecular interactions such as putative linkages between the cytoplasmic expression of junc-

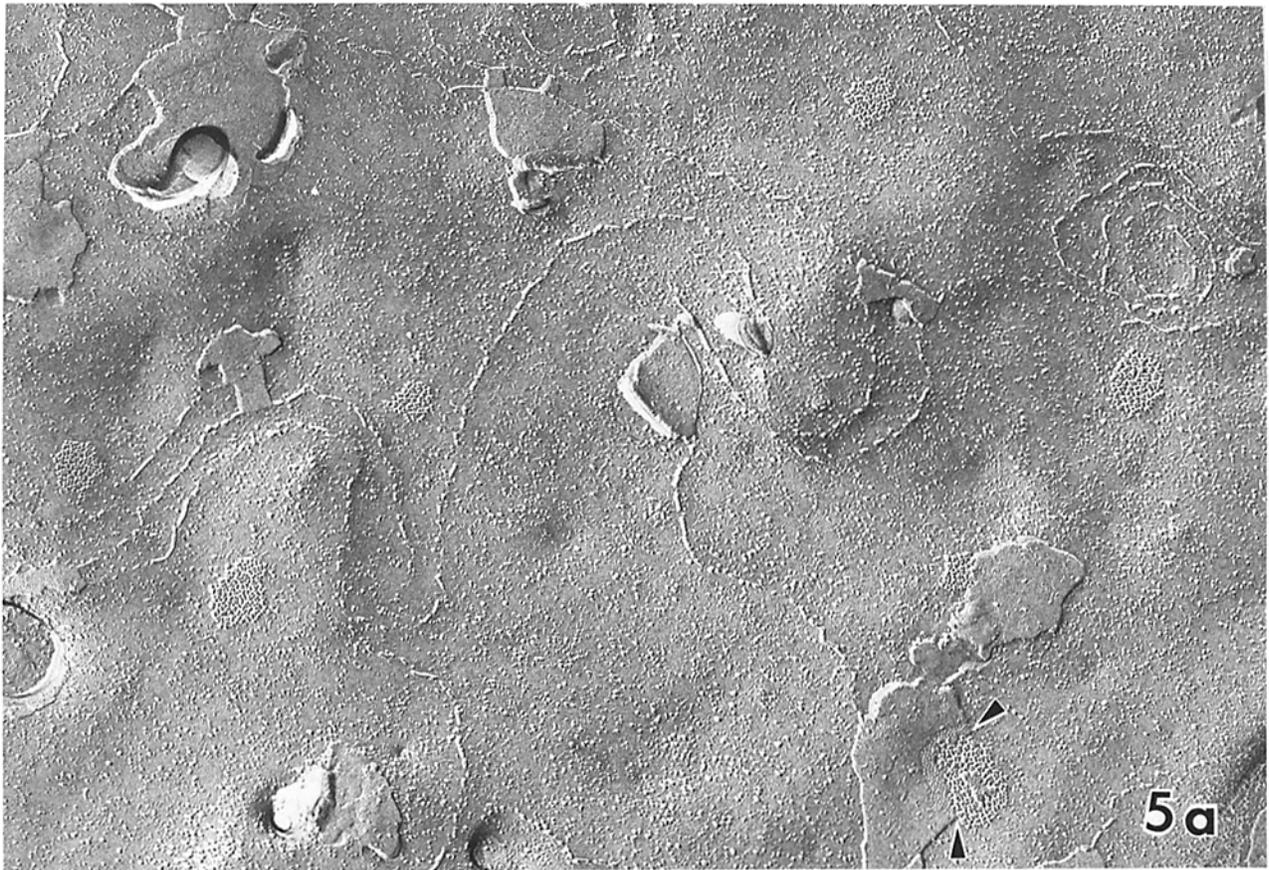


FIGURE 5 Cytochalasin B (a) or colchicine (b) induces the assembly of gap junctions at 37°C or at room temperature. At the transition between P and E faces from adjoining plasma membranes, the complementary aspects of a fractured gap junction (a, arrowheads) are evident and attest to its intercellular nature. a, $\times 45,000$; b, $\times 50,000$.

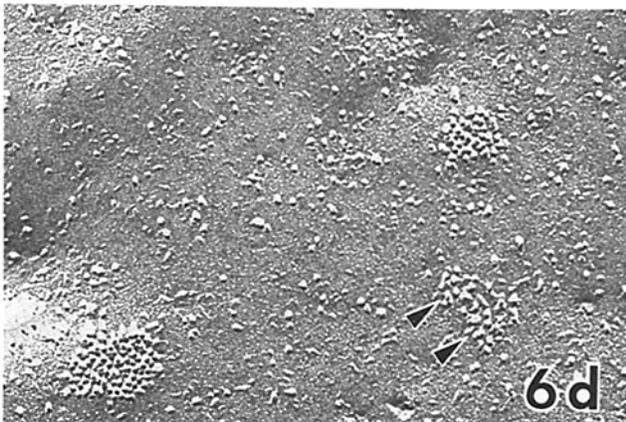
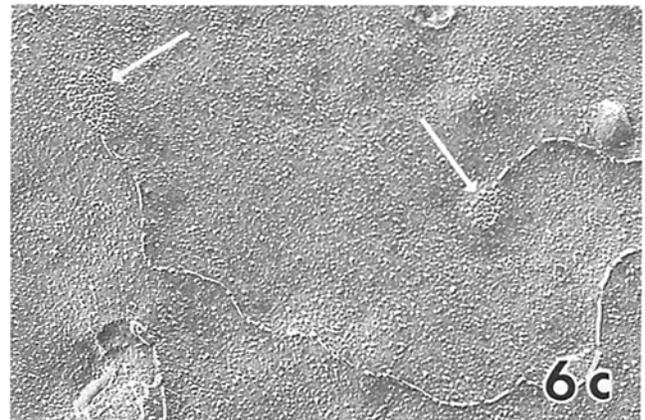
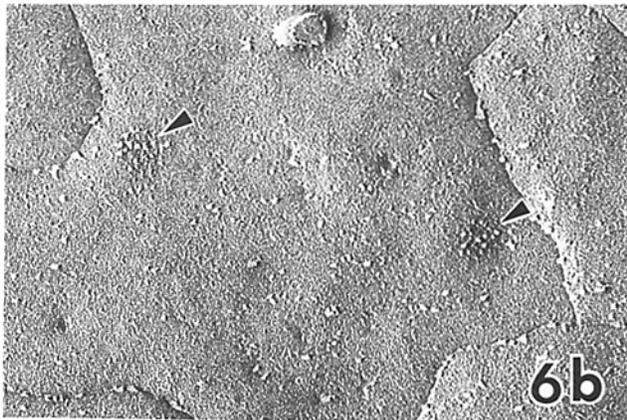


FIGURE 6 Clusters of irregular particles and rugosities (*a, b, d, e*, arrows, arrowheads) coexist frequently with small gap junctions. As small gap junctions, they are round or oval, sometimes also positioned at the tip of tight-junction strands (arrow; compare with *c*). Over E faces the aggregates have less particles (*b*, arrowhead). Aggregates composed of larger particles and fewer rugosities also coexist with gap junctions (*d* and *e*, arrowheads). Texture of the clusters resembles that of desmosomes at the apical band (see Fig. 2, asterisk) but the clusters are smaller and, over P faces, occupy sharply raised areas. Over undifferentiated areas of the plasma membrane the pattern of distribution of intramembrane particles remains undisturbed. *a*, $\times 50,000$; *b*, $\times 60,000$; *c*, $\times 45,000$; *d*, $\times 95,000$; *e*, $\times 95,000$.

tional precursor proteins and microtubules and/or microfilaments. The situation appears more complex, however, because the action of either colchicine or cytochalasin is, by itself, sufficient to release the controls that prevent migration and assembly of gap-junction precursor molecules. The lower number of gap junctions induced by cytochalasin B as compared with that induced by cold or by colchicine could be due to the indirect action of microfilaments, possibly through microtubules, on the migration of the gap-junction precursor molecules. After assembly, the gap junction may remain free from cytoskeletal control. This is indicated by the recent observation that the cytoplasmic surface of gap junctions is unusually smooth, contrasting with the filamentous texture of adjacent areas of nonjunctional membrane (13, 25).

Observation of large, rounded particles in the vicinity of gap junctions led to the proposal that gap junctions are formed by gradual accretion of junctional particles (1, 7, 19). The presence of small clusters of particles that we observed in coexistence with small gap junctions suggests an alternative mechanism. The clusters have the same size, shape, and pattern of distribution as small gap junctions, and even their positional relationship to the tight-junction strands is similar. It is possible that these clusters are in some way related to intermediate stages in the assembly of gap junctions. An alternative assembly mode can be envisaged where, instead of accretion by complete connexons, gap-junction precursor molecules congregate at specific sites, first, and then undergo the physical changes and/or chemical modulation processes that transform these particle clusters into well-defined aggregates of gap-junction connexons. Our experiments cannot define, however, at which point the phenomena of intercellular recognition—which must precede the formation of a structurally and functionally complete gap junction—occur. Do pairs of gap junction precursors from apposing cells mutually recognize one another, first, and then migrate to their final junctional site; or, vice-versa, Does migration to specific sites precede intercellular recognition? In either case, the presence of putative prejunctional aggregates of gap-junction precursor proteins would favor their transformation *en masse* into a gap junction: i.e., the final stages in the construction of the communicating junction take place only after convergence of the precursor molecules to the junctional site. It is not difficult to accept that such a process must involve profound conformational changes; these probably occur during formation of the connexon, i.e., the hexamer with its central hydrophilic channel. The wall in such channels must offer a high electric resistance that isolates the polar contents of the channel from the extracellular environment traversed by the connexon; i.e., the formation of a hydrophilic channel must require not only the structural resources for the intercellular passage of ions and molecules but also the means necessary to negate their exchange with the extracellular environment.

While gap junctions assembled *in vitro* appear structurally intact, their functional competence remains to be ascertained. Another area that remains intriguing and unexplored deals with the ontogenetic and structural relationships as well as functional "linkages" that, we believe, exist between gap and tight junctions and, possibly, desmosomes. This relationship appears complex and asymmetric: for instance, tight-junction strands can be induced to proliferate independent of gap-junction assembly; but the converse—assembly of gap junc-

tions in the absence of tight-junction proliferation—has not been achieved. The investigation of these probable relationships is the object of our current research.

We thank Drs. Maria-Luiza Barbosa, Shelby Berger, and A. P. Aguas for discussions and critical reading of the manuscript. We thank Mr. Clifford Parkison for excellent technical assistance.

Received for publication 10 November 1982, and in revised form 7 January 1983.

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