Structure of Rapidly Frozen Gap Junctions

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ABSTRACT  The structure of gap junctions in the rabbit ciliary epithelium, corneal endothelium, and mouse stomach and liver was studied with the freeze-fracturing technique after rapid freezing to near 4°K from the living state. In the ciliary epithelium, the connexons were randomly distributed, separated by smooth membrane matrix. In the corneal endothelium, both random and crystalline arrangements of the connexons were observed. In the stomach and liver, the connexons were packed but not crystalline. Experimental anoxia or lowered pH caused crystallization of the connexons within 20-30 min. In the ciliary epithelium, the effects of prolonged anoxia or low pH could not be reversed. In addition, invaginated or annular gap junctions increased in number, but their connexons were usually distributed at random. Rapid freezing thus demonstrates that gap junctions of different tissues are highly pleomorphic in the living state, and this may explain their variations in structure after chemical fixation. The slow time-course and irreversibility of the morphological changes induced by prolonged anoxia or low pH suggest that connexon crystallization may be a long-term consequence rather than the morphological correlate of the switch to high resistance.

When gap junctions are chemically fixed and subsequently analyzed with the freeze-fracturing technique, the clustering of their subunits (particles or connexons) in the plane of the membrane varies in different tissues and often in different cells of the same tissue: hexagonal packing and complete randomness are the extreme configurations, but a great variety of intermediate arrangements have been described (4, 10, 20). The functional correlate of these variations is not known, but it seems unlikely that they could reflect differences in the resistance state of the junctions. In fact, it was shown that aldehyde fixation abolishes coupling, and therefore freeze-fractured gap junctions in fixed tissues are probably visualized in their high-resistance state (3). Nevertheless, differences in the degree of clustering of the connexons were observed in tissues exposed to uncoupling agents before fixation as compared with their untreated controls (12, 21, 22); thus, it was proposed that the switch to high resistance is accompanied by movement of the connexons in the plane of the membrane and their lateral association in a compact, crystalline lattice. There are exceptions, however, to this behavior, because uncoupling agents did not seem to affect the state of connexon aggregation in the gap junctions of the lens (11).

These observations raise important questions on the structure of gap junctions in vivo, and on the time-course of crystallization and its precise relationships with uncoupling. Such questions, however, can only be answered if the structure of gap junctions can be analyzed and experimentally manipulated in absence of chemical fixation. For this reason, we used the technique of rapid-freezing to near 4°K (14, 28) on a variety of tissues. With this method, freezing occurs within milliseconds and reveals the unfixed state of biological specimens with minimal ice crystal distortion (14). The structural effects of treatments known to cause the switch of the junctions to their high-resistance state, such as anoxia and low pH, were also investigated. To ensure good freezing, we selected, for this study, tissues in which a large number of gap junctions is present at a distance of 15 μm or less from a free surface.

MATERIALS AND METHODS

Animals and Tissues

Experimental tissues were (a) epithelium of both ciliary and iridal processes of New Zealand (albino) and Dutch Belted (pigmented) rabbits (in the text it will be referred to as ciliary epithelium), (b) corneal endothelium of albino rabbits, (c) stomach and liver of albino mice (strain CD-1, Charles River Laboratories, Cambridge, Mass.).

Solutions

The basic incubation solution contained 120 mM NaCl, 5 mM KCl, 25 mM NaHCO3, 0.8 mM Na2HPO4, 0.1 mM NaH2PO4, 1 mM MgSO4, 2 mM CaCl2, 10 mM glucose (after reference 2, slightly modified). (a) O2-medium: the basic solution was continuously gassed with 95% O2/5% CO2, pH 7.4. (b) N2-medium: the basic solution was continuously gassed with 95% N2/5% CO2, pH 7.4. (c) Low-pH medium: the basic solution was continuously gassed with 40% O2/60% CO2, pH 6.4. As a control medium, the basic solution was gassed with 40% O2/5% CO2/55% N2, pH 7.4.

Freezing and Specimen Preparation for Electron Microscopy

Freezing was done with the apparatus described by Heuser et al. (14), in which the tissue sample is dropped onto a copper block cooled to near 4°K by liquid
helium. After freezing, the tissue blocks were stored in liquid nitrogen and finally fractured and replicated at −115°C with carbon-platinum in a Balzers 301 apparatus (Balzers Corp., Nashua, N. H.). Part of the specimens were freeze-substituted in 10% OsO₄ in acetone, stained in the block with 5% uranyl acetate in absolute methanol, embedded in Epon-Araldite, and sectioned (J. E. Heuser and T. S. Reese, personal communication). Micrographs were taken with a Jeol 100-B electron microscope. All illustrations have approximately the same orientation, with the shadow direction from bottom to top.

Experimental Design
Details of the experiments are specified in Results. Each experiment was repeated a minimum of four times in each tissue.

RESULTS

Rabbit Ciliary Epithelium

The epithelium of the ciliary and iridal processes of the rabbit eye consists of a pigmented and a nonpigmented layer of cells, which are joined at their apices and sandwiched between two basal laminae. All cells of the ciliary epithelium are interconnected by gap junctions, but these are especially numerous at the interface between pigmented and nonpigmented cells, where they occupy 8% of the apical plasmalemma (24). This interface lies at a distance of 5–15 μm from the free surface of the epithelium and is, therefore, included within the region that can be optimally frozen by the technique of Heuser et al. (14). Nonpigmented cells are also connected by a simple but continuous zonula occludens. Dissection of the ciliary epithelium is time-consuming; thus, to avoid drying and the effects of a prolonged interruption of the blood supply, the enucleated eye was hemisected and rapidly removed and the anterior segment was trimmed into sections 3 mm wide. This whole procedure was completed in <60 s. After an incubation of 5–60 min in O₂ medium, the specimens were frozen at 4°K and transferred to liquid nitrogen. Upon freezing, the anterior segment was trimmed into strips 3 mm wide. This whole procedure was completed in <60 s. After an incubation of 5–60 min in O₂ medium, the specimens were frozen at 4°K and transferred to liquid nitrogen. Upon freezing, the epithelial layer of cells was hemisected and immediately immersed in O₂-medium at 37°C. Lens, ciliary zonule, and remnants of the vitreous body were rapidly removed and the anterior segment was trimmed into strips 3–5 mm wide. This whole procedure was completed in <60 s. After an incubation of 5–60 min in O₂ medium, the specimens were frozen at 4°K and transferred to liquid nitrogen. Upon freeze-fracturing, the gap junctions between epithelial cells had a most unusual appearance: on the P face, the junctional particles were set far apart from one another; they appeared randomly distributed and were separated by broad expanses of smooth membrane matrix (Fig. 1). The particles had uniformly large diameter (8.74 ± 0.57 nm, SD, 25 measurements), except for a small number of tiny protrusions, 3–5 nm in diameter, of unknown significance. The center-to-center distance of the particles varied greatly, and only exceptionally did they appear in contact with one another. The junctional aggregates, in spite of their loose appearance, could be easily distinguished from the surrounding, nonjunctional membrane because of the large, uniform diameter of their particles. In addition, they were frequently observed in register with disorderly arrays of pits on the E face of the adjoining cell membrane across a narrowing of the intercellular space (Fig. 1, inset A). The gap junctions at the interface between pigmented and nonpigmented cells were frequently associated with short tight-junction strands (Fig. 1), and the gap junctions between adjacent nonpigmented cells were usually inserted within the meshwork of the zonula occludens. As reported in other freeze-fractured, unfixed tissues (25, 27), the tight-junction strands fragmented upon fracturing and appeared unequally partitioned between P- and E-fracture faces.

In freeze-substituted, thin-sectioned specimens, gap junctions were easily distinguished as flat pentalaminar fusion plaques (Fig. 1, inset B), an appearance common to gap junctions treated with osmium tetroxide as a primary fixative (6).

The effects of anoxia on the structure of ciliary gap junctions were tested by transferring the specimens to N₂-medium for variable time intervals after an initial 5- to 30-min incubation in O₂-medium. After 5–15 min of anoxia, the junctional particles began to aggregate into small clusters or short rows (Fig. 2). By 20–30 min, all junctions condensed into tightly packed aggregates without appreciable amounts of interstitial matrix (Fig. 3). The diameter of the particles did not change significantly (8.61 ± 0.70 nm, SD, 25 measurements) and their center-to-center spacing was similar to their diameter. The bidimensional crystal thus generated exhibited hexagonal symmetry. Many crystalline junctions appeared gently curved. Furthermore, a few junctions were deeply invaginated into the cytoplasm of one of the partner cells; usually, however, their particles were widely dispersed and randomly distributed or formed small clusters and short rows (Fig. 4). Freeze-substitution and thin-sectioning confirmed that the cytoplasm of the epithelial cells contained numerous invaginated or annular (1, 19) gap junctions (Fig. 5).

To test whether the effects of anoxia were reversible, tissue samples were incubated in O₂-medium for 5 min, transferred to N₂-medium for 15–30 min, and finally returned to O₂-medium for 5–60 min. As a rule, the crystalline appearance typical of the anoxic state could not be reversed; in two specimens, however, which were exposed to relatively short periods of anoxia (15 and 20 min), junctions had particles widely dispersed (Fig. 6) or appeared as a constellation of small clusters separated by broad expanses of smooth membrane matrix.

The effects of exposure to low pH were tested by incubating the tissue samples in O₂-medium for 5–30 min, followed by 5–30 min at pH 6.4. The structural changes were identical as with anoxia and had the same time-course: after 20–30 min, all junctions at the cell surface exhibited a tightly packed hexagonal configuration (Fig. 7) and were frequently curved. Occasionally, junctions appeared deeply invaginated; their particles, however, were frequently dispersed. Recovery from exposure to low pH was studied by returning the samples to O₂-medium at pH 7.4 for 5–60 min: again, junctions retained their crystalline configuration. Because low pH was obtained by gassing the bicarbonate medium with a 40% O₂/60% CO₂ mixture, the effects of a lower oxygen concentration were tested by incubating tissue samples for 5–30 min in O₂-medium, followed by 5–30 min in a medium at pH 7.4 gassed with 40% O₂/5% CO₂/55% N₂. The influence of this treatment on gap junctions was slight; there was a tendency of the particles to aggregate into small clusters or short rows, but they did not crystallize (Fig. 7, inset). Because of the long time-course of the reversibility experiments, we were unable to discriminate between reutilization and de novo assembly of gap junctions. Thus, reversibility experiments were not tried in other tissues.

Rabbit Corneal Endothelium

Corneas were rapidly transferred to and trimmed in O₂-medium at 37°C. The tissue blocks were subsequently incubated in this medium from 5 to 30 min, frozen, and freeze-fractured. Unlike the situation in ciliary epithelium, the gap junctions between endothelial cells showed great variation in structure; in some junctions, the particles were widely dispersed and randomly distributed (Fig. 8), in others they were hexagonally packed (Fig. 10). Intermediate forms of aggregation were also seen, characterized by a tendency of the particles to form small clusters or rows (Fig. 9). Invaginated junctions with
Rabbit ciliary epithelium: 10 min O₂-medium. After rapid freezing from the living state, the gap junctions that connect pigmented and nonpigmented cells have particles distributed at random and separated by broad expanses of smooth membrane matrix. On the P face, the junctional aggregates can be distinguished from the surrounding, unspecialized plasmalemma because of the large, uniform diameter of their particles and because they are often in register with disorderly arrays of pits on the E face of the adjoining cell membrane (Inset A, arrowheads; the inset is an enlargement of the area labeled by an asterisk). The tight-junction strands (tj), which are associated with the periphery of some gap junctions, fragment upon fracturing and appear unequally partitioned between P- and E-fracture faces. In freeze-substituted, thin-sectioned specimens, gap junctions appear as flat, pentalaminar plaques of membrane fusion (Inset B, arrowheads). × 143,000; A, × 208,000; B, × 103,000.
widely dispersed particles were occasionally present in freeze-fracture replicas. We had the impression that although the morphology of gap junctions varied in different cells, all junctions in the same cell had similar configuration. As a result of a 30-min treatment with N2-medium, all junctions became hexagonally packed (Fig. 11).

Mouse Stomach

Fragments of the wall of the stomach were either rapidly dissected from the anesthetized animal, trimmed and frozen within 15–20 s from suspension of the blood supply, or incubated for 5–60 min in O2-medium before freezing. In both instances, the particles of the gap junctions between surface mucous cells appeared clustered, but they did not display hexagonal packing (Fig. 12). This short-range disorder disappeared after a 20-min incubation in N2-medium, for they crystallized into a regular hexagonal lattice (Fig. 12, inset).

Mouse Liver

The caudate lobe of the liver was gently dissected from the anesthetized animal and frozen within 15 s from removal. Hepatocyte gap junctions had the same configuration as in chemically fixed, conventionally frozen specimens: their particles were clustered without tight hexagonal packing (Fig. 13).

DISCUSSION

This paper reports that the appearance of gap junctions in living, unfixed specimens which were rapidly frozen to near 4°C varied considerably in different organs: in the rabbit ciliary epithelium, the connexons were distributed randomly and separated by smooth membrane matrix. In the rabbit corneal endothelium, both the random or amorphous arrangement and the closely packed or crystalline configuration were observed. In the unfixed, rapid-frozen stomach and liver of the mouse, the distribution of the connexons was indistinguishable from that in fixed specimens, in which gap junctions are packed but not crystalline (7, 21). The significance of these variations in gap junction structure in different organs remains obscure: a crucial issue is whether they exist in vivo or reflect different sensitivities of the tissues to the manipulations and traumas that preceded freezing. Because crystallization of the junctions requires minutes of anoxia or incubation at low pH, we do not believe that extensive rearrangement of the connexons could take place in the samples of stomach and liver during the few seconds interval elapsing between excision and freezing. On the other hand, gap junctions of stomach mucous cells appeared clustered both in specimens frozen immediately after removal and in those incubated in vitro for variable time intervals. Thus, it seems unlikely that the dispersed configuration of the junctions in the ciliary epithelium was caused by the incubation in vitro, a procedure we adopted to prevent drying and the effects of a prolonged interruption of the blood supply. Nevertheless, we cannot rule out the possibility that changes may occur at the moment of excision of the tissue, for in the ciliary epithelium, stomach, and liver, the connexons of all gap junctions appeared in the same state of aggregation whereas in the rabbit corneal endothelium, crystalline and amorphous junctions coexisted. This variation may reflect an exquisite corneal sensitivity to trauma or dynamic, ongoing processes of insertion and removal of the junctions.

Gap junctions not only differ in their native structure in different epithelia but also vary in their response to aldehyde fixation. Gap junctions in stomach and liver are scarcely affected by fixatives, whereas those in the corneal endothelium and ciliary epithelium tend to crystallize into linear domains of hexagonally packed particles separated by aisles of smooth membrane matrix (18, 24), a phenomenon observed in other tissues as well, such as the ovary (1).

When the tissues were rendered anoxic or the pH of the bathing medium was lowered, the connexons aggregated into a bidimensional, hexagonal crystal. Lowering of the intracellular pH is known to switch gap junctions to their high-resistance state (26), and anoxia may have a similar effect (21). However, the relationship between crystallization of the connexons and cells’ uncoupling remains unclear. In the ciliary epithelium, connexons were partially clustered at 10 min and became fully aggregated after 20–30 min. Furthermore, redisperal of the fully aggregated junctions was never observed, suggesting that crystallization is an irreversible event. In the few instances in which connexons appeared dispersed at the end of the recovery experiments, the period of anoxia was relatively short; furthermore, there was ample time for synthesis and insertion of new junctions on the cell surface (9). Electrophysiological studies have shown that exposure to low pH

![Figure 2](image-url) Rabbit ciliary epithelium: 10 min O2-medium, 8 min N2-medium. As a result of a brief period of anoxia, the particles of gap junctions begin to aggregate into small clusters and short rows. In this and the following figures, the P- and E-fracture faces are labeled P and E, respectively. X 150,000.

![Figure 3](image-url) Rabbit ciliary epithelium: 10 min O2-medium, 30 min N2-medium. With longer periods of anoxia, the particles condense into a tightly packed aggregate without appreciable amounts of interstitial matrix. X 190,000.

![Figure 4](image-url) Rabbit ciliary epithelium: 10 min O2-medium, 30 min N2-medium. In anoxic specimens, some gap junctions become deeply invaginated (IC). Note that the junctional particles are either distributed at random or form small clusters, in striking contrast with the crystalline configuration of the junctions that reside at the cell surface. The arrowhead indicates the neck of the invagination. X 101,000.

![Figure 5](image-url) Rabbit ciliary epithelium: 10 min O2-medium, 45 min N2-medium. Freeze-substitution and thin-sectioning demonstrate that anoxia causes a striking increase in the number of invaginated or annular gap junctions (arrows). X 67,000.

![Figure 6](image-url) Rabbit ciliary epithelium: 5 min O2-medium, 15 min N2-medium, 10 min O2-medium. This micrograph illustrates one of the few examples in which gap junctions had widely dispersed particles when the anoxic specimen was returned to an oxygenated medium. X 108,000.

![Figure 7](image-url) Rabbit ciliary epithelium: 20 min O2-medium, 20 min at pH 6.4. After exposure to low pH, gap junctions become crystalline and frequently curve. Inset, 20 min O2-medium, 20 min in medium gassed with 40% O2/5% CO2/55% N2. In control specimens treated with low oxygen medium at pH 7.4, the junctional particles show a tendency to form small clusters or short rows separated by smooth membrane matrix, but they do not crystallize. X 107,000; inset, X 128,000.

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FIGURES 8-10  Rabbit corneal endothelium: 10 min O₂-medium. In some gap junctions, the particles are widely dispersed (Fig. 8);
other junctions are more packed but do not exhibit hexagonal symmetry (Fig. 9). Finally, many junctions are found that display a
crystalline configuration (Fig. 10). The arrowheads indicate tight junction strands associated with the gap junctions. Fig. 8,
× 158,000; Fig. 9, × 94,000; Fig. 10, × 116,000.

FIGURE 11  Rabbit corneal endothelium: 10 min O₂-medium, 30 min N₂-medium. All gap junctions become crystalline with
anoxia. The arrowheads indicate a tight-junction strand associated with the gap junction. × 196,000.

FIGURE 12  Mouse stomach: 10 min O₂-medium. The particles of the gap junctions between surface mucous cells are clustered,
but they do not exhibit hexagonal packing. Inset, 10 min O₂-medium, 20 min N₂-medium. Anoxia induces crystallization of the
gap junctions. × 133,000; inset, × 150,000.

FIGURE 13  Mouse liver: rapid freezing 15 s after removal from the anesthetized animal. The gap junctions between hepatocytes
have the same appearance as in chemically fixed specimens: their particles are clustered but not hexagonally packed. × 126,000.
abolished coupling within 20 s in fish blastomeres (5), 1 min in mouse pancreas acinar cells (15), and 2.5 min in amphibian embryos (26); in all three instances, coupling could be restored on a time-scale of seconds or minutes. These discrepancies between structural and physiological events raise doubts that crystallization of the connexons represents the structural correlate of uncoupling. It is interesting to note that in the tissues studied here, uncoupling agents have a different effect than chemical fixation, because anoxia and low pH, but not aldehyde treatment, transform gap junctions into tightly packed hexagonal aggregates. Perhaps the fixative interferes with the natural tendency of the uncoupled junctions to condense slowly into a faultless crystal.

The chemical events responsible for crystallization of gap junctions are unknown; an attractive hypothesis is that uncoupling is followed by conformational changes in the connexons and subsequent increase in their mutual affinity. Alternatively, it may be a patching phenomenon, mediated by cross-linking of cytoplasmic components situated on the inner aspect of the junctional membrane.

Both anoxia and low pH caused a dramatic increase in invaginated or annular gap junctions; surprisingly enough, however, most invaginated junctions were amorphous. Either crystallization is not a prerequisite for invagination or crystalline junctions disperse upon invagination. For this very same reason, it seems unlikely that the gentle curvature of many crystallized junctions could be a prelude to their invagination. Perhaps curving, as opposed to invagination, is a purely physical phenomenon that results from the fact that contraction of the lattice does not occur synchronously in both adjoining membranes (4). Invagination, on the other hand, may represent a biological response to uncoupling and one of the means of gap junction disposal. In fact, it seems unlikely that invagination could reflect insertion of newly formed junctions on the cell surface, for there is evidence that (a) neoformation of gap junctions is not associated with presence of invaginated junctions (8, 16), (b) invagination follows interruption of the blood supply (24), and (c) it may ultimately lead to junction internalization and digestion (19, 24).

In conclusion, the present study demonstrates remarkable variations in the organization of gap junctions of different tissues when they are rapidly frozen from the living state; probably, this pleiomorphism of fixed junctions explains their different behavior in response to fixation. The structural diversity of native gap junctions is not surprising, if one considers that junctional proteins extracted from different sources, such as liver, heart, and lens, show biochemical differences (11, 13, 17). Treatment with uncoupling agents causes crystallization or invagination of the junctions, but the correlation between physiological and morphological events remains to be elucidated. Probably, a crystalline junction is in its high-resistance state, but random distribution of the connexons does not necessarily mean that they are traversed by patent channels.

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