CARDIAC GAP JUNCTION CONFIGURATION AFTER AN UNCOUPLING TREATMENT AS A FUNCTION OF TIME

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ABSTRACT

Rabbit ventricle either was fixed in glutaraldehyde without injury (control) or was injured before fixation, presumably causing electrical uncoupling of the gap junctions. All tissue was then processed for freeze-fracture. Replicas of control gap junctions exhibited irregular packing of the P-face particles and E-face pits. Average center-to-center spacing of the particles was 10.5 nm. Tissue fixed 1-5 min after injury showed clumping of gap junctional particles and pits. Within the clumps, the particles and pits were hexagonally packed and the center-to-center spacing of the particles averaged 9.5 nm. In tissue fixed 15-30 min after injury, the clumps of gap junctional particles had coalesced into a homogeneous structure in most junctions. The packing of the particles and pits was hexagonal and the spacing of the particles averaged 9.5 nm. A few pieces of rabbit atrium were frozen without prior fixation or cryoprotection to try to assess the effect of glutaraldehyde fixation on gap junction structure. In this tissue the gap junctional particles were irregularly packed and their spacing averaged 10.0 nm.

KEY WORDS cardiac muscle gap junctions freeze-fracture junctional particles uncoupling

Gap junctions are generally recognized as the sites of electrical coupling between cardiac muscle cells (5, 13, 18, 21, 22). It might be expected, therefore, that the structure of cardiac gap junctions would change when the cells become electrically uncoupled. Unfortunately, gap junctions electrically uncoupled by injury appeared similar in electron microscope studies of sectioned cardiac muscle to those of uninjured control cells (2, 4). However, it has been possible, using the freeze-fracture technique, to demonstrate a change in mammalian cardiac gap junctions after an uncoupling treatment.

Peracchia and Dulhunty (25) have shown a decrease in beading periodicity and gap junction width in crayfish gap junctions uncoupled by EDTA or dinitrophenol. Freeze-fracture replicas of similarly treated junctions showed an increase in regularity of particle and pit packing and a decrease in spacing between particles and pits. Peracchia (23) also has reported similar kinds of changes in replicas of gap junctions from rat liver and stomach after uncoupling procedures. Furthermore, a decrease in particle diameter in the altered junctions has been described (23–25).

In the present study, a decrease in particle and pit spacing and an increase in regularity of particle packing is shown to occur in rabbit heart gap junctions presumed uncoupled by injury. Furthermore, it is demonstrated that shortly after uncoupling treatment, the gap junctional particles were aggregated into clumps, leaving particle-free areas within the junction. Later, these clumps coalesced into a homogeneous compact unit in most junctions.
MATERIALS AND METHODS

Adult rabbits, 3-5 kg in weight, were anesthetized with Nembutal (Abbott Laboratories, North Chicago, Ill.), and the hearts were rapidly excised and placed in an oxygenated Tyrode's solution (152.5 mM Na\(^+\), 2.7 mM K\(^+\), 1.8 mM Ca\(^{2+}\), 148.3 mM Cl\(^-\), 12.0 mM HCO\(_3\)\(^-\), 0.4 mM H\(_2\)PO\(_4\)\(^-\), and 5.5 mM glucose at pH 7.1). The experiments were carried out at room temperature because Weidmann (34) has reported that some ventricular trabeculae underwent progressive contracture at 37°C, but could be maintained for long periods at room temperature.

**Fixed Control Tissue**

The entire ventricle was cut into 2- to 3-mm slices, with the cuts made perpendicular to the interventricular septum. The largest of these slices, that from near the atrioventricular border, was placed immediately into 1.2% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. The rest of the slices were used for experimental tissue (see below). After 30-min fixation, the slice of ventricle was further cut into 2- to 3-mm cubes. Each cube had the endocardium at one face, the epicardium at the opposite face, and cut surfaces at the other four faces. These cut surfaces would be expected to contain injured cells because the cuts were made before fixation (two faces) or after only a brief fixation (the other two faces). Therefore, after an additional 30- to 60-min fixation, the original cut surfaces, with their injured cells, were removed by trimming an ~0.5-mm-thick slice of tissue away from each of the original cut faces of the cubes. The endocardium and epicardium were also trimmed away so that the tissue blocks were reduced to ~1-mm cubes. The newly cut surfaces contained cells which were presumed to be uninjured because they were cut after being fixed. After a total fixation of 1.5-2.5 h, the blocks were rinsed overnight in 0.15 M cacodylate buffer at room temperature. The tissue blocks were placed in Balzers' specimen carriers (Balzers' Corp., Nashua, N.H.), frozen in Freon 22 and then placed in liquid nitrogen. The frozen specimens were fractured at ~100°C in a vacuum of ~2 × 10\(^{-6}\) torr in a Balzers' 360 M freeze-fracture apparatus equipped with an electron beam evaporation gun. Replication began within 5 s of the last cut. In some cases, tissue was stored for several days at 4°C in either the 0.15 M cacodylate buffer or the 25% glycerol solution before further processing. The results were the same for all treatments.

**Unfixed Control Tissue**

To assess the effects of glutaraldehyde fixation on the structure of gap junctions, a few pieces of rabbit atrium were frozen directly in Freon 22 without prior fixation or cryoprotection. To do this, a piece of atrium, ~3-4 mm across, was removed from the atrial wall. The whole piece was placed on a Balzers' specimen holder, epicar-
FIGURE 1  Gap junctions from fixed control tissue. Note the irregular packing of the particles on the F face and pits on the E face. Average center-to-center spacing of closely adjacent particles was 10.5 nm. Bar, 0.1 μm. × 116,000.
ticles, and the E face by corresponding pits. In the majority of junctions from this tissue (75 out of 86), the junctional particles and pits were gathered in a homogeneous array. The packing of the particles and pits was irregular, but small areas of hexagonally packed particles were sometimes seen (Fig. 1a–d). The spacing between the particles was not uniform, but the average center-to-center spacing of closely adjacent, clearly resolved particles in 32 gap junctions was 10.5 ± 0.3 nm (Fig. 2).

When both P-face particles and E-face pits were measured from the same junction, the spacing of the pits was always less than the spacing of the particles. Good consistent measurements of the center-to-center spacing of the pits were not obtained because of the difficulty in accurately determining the center of the pits. However, the average center-to-center spacing of the pits appeared to be ~1 nm less than that of the particles.

In 11 out of 86 junctions, the particles were somewhat clumped rather than forming a homogeneous structure. In all of these junctions, the particles and pits were hexagonally packed.

Unfixed Control Tissue

Gap junctions from unfixed, uncryoprotected tissue were similar to those of the fixed controls in that the packing of the junctional particles was predominantly irregular, but small areas of hexagonal packing were seen within the junctions (Fig. 3). The interparticle spacing was less uniform than in the fixed control tissue. The center-to-center spacing of closely adjacent P-face particles averaged 10.0 nm ± 0.3 nm for eight junctions. As in the fixed controls, the E-face pits were irregularly arranged, and their spacing was generally less than that seen for particles.

In a total of 34 junctions in this group, 26 were homogeneous structures with irregularly packed particles and pits, 6 were mostly homogeneous with irregularly packed particles (but distinct clumps of hexagonally packed particles and pits were seen within them) and 2 were entirely formed by clumped, hexagonally packed particles.

Experimental Tissue, Fixed 1–5 Min After Injury

At first appearance, most gap junctions from tissue fixed a short time after cutting injuries appeared to be disrupted. The particles were found in small clumps with smooth particle-free areas within the junctions (Fig. 4a and b). On close examination, however, the particles within the clumps were seen to be more tightly and regularly packed than in the control junctions. The average center-to-center spacing of closely adjacent particles in 10 junctions was 9.5 ± 0.4 nm (Fig. 2). As a rule, the particle packing was hexagonal as compared to the irregular packing seen in the controls. This difference in packing was most striking when comparing the E-face pits of the injured tissue to the control samples (Figs. 1a and 4c). As in the control gap junctions, the spacing of the E-face pits was less than the spacing of the particles within a given junction. The average center-to-center spacing of the E-face pits in 13 junctions was 8.2 ± 0.3 nm. Unlike that in the control junctions, the spacing of the pits was easily measured because of their very regular arrangement in rows.

The degree of particle clumping in these junctions was quite variable. In a total of 42 junctions in this group, 15 had obvious clumping (Fig. 4a and b) while 27 had less marked clumping (Fig. 4c). In no junctions did the particles form a completely homogeneous array.

Experimental Tissue, Fixed 15–30 Min After Injury

While some gap junctions from tissue fixed 15–30 min after injury were similar to those fixed 1–
5 min after injury (Fig. 5c), most of them differed in that the particles formed a homogeneous structure with hexagonally packed particles and pits (Fig. 5a–b). From measurements made on 11 junctions, the average center-to-center spacing of closely adjacent particles in these junctions was 9.5 ± 0.5 nm (Fig. 2). The center-to-center spacing of the E-face pits in nine junctions was 8.3 ± 0.2 nm.

In a total of 48 junctions in this group, 34 were homogeneous structures with hexagonally packed particles and pits (Fig. 5a and b), 10 showed a slight degree of clumping, and in 4 the clumping was quite marked (Fig. 5c).

DISCUSSION
Because it was not possible to knowingly make electrophysiological measurements on the same cells as those used for freeze-fracture studies, it was important to treat the tissue in such a way that
FIGURE 4 Gap junctions from tissue fixed 1-5 min after injury. Average particle spacing was 9.5 nm. Obvious clumping of junctional particles was present in some junctions from this tissue (4a and b), but the clumping was less marked in others (4c). Hexagonal packing was seen within the clumps. Bar, 0.1 μm. × 116,000.
most, if not all, of the cells fractured were in the desired functional state. An attempt to do this was the basis for the methods chosen.

By a careful trimming of the blocks of control tissue during and after fixation, an attempt was made to remove all cells injured during dissection. That this attempt was largely successful is supported by two observations. First, the majority of junctions from this tissue were similar in structure to junctions from perfusion-fixed tissue (23) where none of the cells would be expected to be injured.

Second, when blocks of control tissue were post-fixed, embedded, and sectioned (Baldwin, unpublished observation), most cells in them appeared uninjured and their myofibrils were in a relaxed state. A few cells, however, did appear injured because their myofibrils had undergone marked contracture. Thus, even though a few injured cells might be expected to have been present in the blocks of control tissue used for freeze-fracture.

![Figure 5](image-url)  
**Figure 5** Gap junctions from tissue fixed 15-30 min after injury. The average particle spacing was 9.5 nm. Most junctions from this tissue were homogeneous (5a and b), but a few had clumped particles (5c). Note the hexagonal packing. Bar, 0.1 μm. × 116,000.
the majority of the control junctions, those with irregularly packed particles in a homogeneous array, are presumed to have been from uninjured cells. It seems likely that those few junctions with clumped, hexagonally packed particles seen in replicas of control tissues were actually from some injured cells not removed by the trimming procedure.

As all tissue was fixed by immersion, it is possible that the cells were somewhat anoxic. However, because Peracchia (23) has shown that gap junctions uncoupled by anoxia are the closely packed, hexagonal configuration, it is reasonable to assume that any anoxia present was not so severe as to uncouple the cells.

Bennett (6) has reported that glutaraldehyde fixation caused an increase in intracellular resistance which was transient in some tissues but permanent in others. Whether the transient increase in resistance was followed by a return to the coupled state (25) or by a secondary artificial type of uncoupling (6) is not known, but the possibility that glutaraldehyde fixation permanently alters gap-junction function must be acknowledged. In any case, junctions that are coupled at the time of fixation behave unlike those that are uncoupled at the time of fixation (3, 23, 24, 25, and this study), and we may conclude that even if glutaraldehyde does cause permanent uncoupling of gap junctions, it does so in a manner different from that seen after cellular injury.

Unfixed, uncryoprotected tissue was used in an attempt to gain information regarding the effects of glutaraldehyde fixation on the structure of gap junctions. Large cytoplasmic ice crystals could be seen in cells of the unfixed tissue (Fig. 3), but the cell membranes appeared to be intact and undamaged. The particle packing pattern in most of the junctions was irregular, similar to those from fixed control tissue. The average interparticle spacing in the unfixed control junctions, however, was not significantly different from that in either the fixed control or the fixed experimental junctions. In spite of the limitations of the conventional freezing technique with unfixed, uncryoprotected tissue, we may at least conclude that the irregular packing of junctional particles in control tissue is not an artifact of glutaraldehyde fixation, because it is seen in unfixed tissues. A recent report of gap junctions in rapidly frozen, unfixed irises (28) indicates that after rapid freezing, junctional particles are irregularly arranged but widely spaced. It is possible that the appearance of the unfixed junctions in this study resulted from cell shrinkage because the particle spacing is less than that seen after rapid freezing.

Those few junctions with clumped, hexagonally packed particles seen in the unfixed control tissue may have been from cells injured (and presumably uncoupled) by the freezing and/or the dissection.

For over a century it has been known that cardiac muscle rapidly heals-over after an injury (14). This healing-over is indicated by the disappearance of the injury potential and is caused by the electrical uncoupling of the injured cells from the uninjured cells (2, 4, 9, 10). Electrical uncoupling after injury has been shown for several types of noncardiac cells also (1, 20, 27, 31). The uncoupling of intercellular junctions after injury is thought to be caused by abnormally high levels of intracellular calcium (9, 11, 20, 32). The work of Peracchia (24) on isolated gap junctions, which shows that particle packing configuration, irregular vs. hexagonal, depends on calcium concentration in the isolation medium, makes a nice connecting link between the uncoupling with calcium hypothesis and the results of this and other morphological studies on uncoupled gap junctions.

Blocks of rabbit ventricular muscle that have been cut in Tyrode's solution, allowed to heal-over, and then fixed, embedded, and sectioned have cells at their surfaces which show marked contracture (Baldwin, unpublished data). This contracture, presumably due to a high intracellular calcium concentration resulting from the injury, was not confined to the cells that were actually cut. A zone of injury 100-200 μm deep extended into the blocks from all surfaces cut in Tyrode's solution before fixation. The spread of injury to cells adjacent to those directly injured has also been reported for other types of cardiac muscle (4, 9, 10, 12). The blocks of ventricular muscle used in this study, therefore, would be expected to have had injured cells for a depth of at least 100 μm from their cut surfaces. As replicas of experimental tissue were obtained from within 100 μm of a cut (injured) surface, it is assumed that they were of injured cells. Because the intracellular calcium levels that cause contracture are similar to those that cause electrical uncoupling in cardiac muscle (35), it seems reasonable to assume that these...
injured cells are also electrically uncoupled.

The average interparticle spacings in junctions from both types of experimental tissue (9.5 nm) were significantly different from the average interparticle spacing from fixed control tissue (10.5 nm) at above the 99% confidence level using Dunn's multiple comparison test. The clumping of junctional particles in tissues fixed 1-5 min after injury varied from marked (Fig. 4a) to slight (Fig. 4c), but none of the junctions had particles in a completely homogeneous array as was often seen in tissues fixed at later times. The reason for this variability is unknown, but the difference in clumping might be explained by the different healing-over times used (1 min vs. 5 min), by variations in junctional location with respect to a cut surface, or may simply be a result of differences in individual cells in their reaction to injury. In any case, some degree of junctional particle clumping seems to be the rule for the short-term reaction of cardiac muscle cells to this type of injury.

While some junctions from tissues fixed 15-30 min after injury also had clumped particles, most of them (71%) were homogeneous structures. This would indicate that the usual pattern of reaction to injury is a clumping of particles into hexagonally packed clusters and then a later coalescence of the clumped particles into a homogeneous, hexagonally packed unit. The presence of some junctions with clumped particles in tissues fixed 15-30 min after injury may be a result of the different healing-over times used (15 min vs. 30 min), or of variations in junctional location with respect to a cut surface, but the possibility that some junctions maintain the clumped arrangement of particles after injury cannot be ruled out. Initial clumping of junctional particles followed by coalescence into a large aggregate has recently been reported for injured, rapidly frozen, unfixed iridial gap junctions (28).

The lack of correspondence of spacing between E-face pits and Mace particles within the same junction is disturbing. It has been postulated that the particles in the membrane in one cell are lined up with the particles in the membrane of the adjacent cell and that during fracturing the pits are formed by the removal of the particles along with the P half of one of the membranes (8, 21, 22). Thus, one would expect the arrangement of the pits to coincide with the arrangement of the particles. Because the spacing of the pits was always less than the spacing of the particles, it is likely that this discrepancy is caused by some plastic deformation of either the pits, the particles, or both during fracturing rather than misalignment of the particles in one cell with respect to the particles in the other. Caspar et al. (7) have studied isolated gap junctions with negative staining and x-ray diffraction. These isolated gap junctions have been exposed to high calcium solutions and the connexon units making up these junctions are, therefore, in the form of a hexagonal lattice. The lattice constant in such junctions varies from 8.0 to 9.0 nm. Comparing this x-ray diffraction and negative-staining data to the finding in this study that hexagonally packed junctional pits are spaced an average of 8.2-8.3 nm apart would indicate that during fracturing it is the particles that undergo plastic deformation and that the pits reflect the more accurate, undeformed picture.

It is interesting to note that the "normal" structure of at least some gap junctions does not include hexagonal packing of the junctional particles as was proposed earlier (17, 21, 22, 30, 33). Furthermore, because both a decrease in particle spacing and an increase in regularity of particle packing have been seen in different tissues with a variety of uncoupling procedures (23, 25, this study), it seems likely that these effects are not unique to these tissues but may be seen whenever gap junctions are uncoupled. The clumping of particles shortly after uncoupling as reported here may also be a common feature. Such clumping was not reported by Peracchia (23) or Peracchia and Dulhunty (25), but their studies involved relatively longer uncoupling procedures and thus may have not shown that stage. Certainly, gap junctions with clumped, hexagonally packed particles are commonly reported in the literature (15, 16, 19, 26, 29), and it is possible that some of these are in fact uncoupled.

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