Cardiac Gap Junctions and Gap Junction-Associated Vesicles: Ultrastructural Comparison of In Situ Negative Staining With Conventional Positive Staining

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By comparing in situ negative staining of mammalian heart muscle using La(NO₃)₃ with conventional positive staining by uranium and lead salts, we showed that 1) the membrane area of rat cardiac gap junctions (GJs) at the intercalated disks is threefold to fourfold greater than previously thought; 2) connexon arrays of cardiac GJ are subdivided into microdomains by connexon-free aisles; 3) profiles of GJ-associated vesicles (GJAVs) of plasmalemmal origin (which are present extracellularly and sharply localized at three extracellular sites) are paired to form GJs with each other and with myocyte plasmalemma; 4) some GJAVs contain arrays of assembled connexons; and 5) myocytes contain intracytoplasmic complexes lying within cylindrical or cigar-shaped membranes and consisting of GJs and multiple vesicles apparently dissociating from these GJs. (Circulation Research 1989;64:501-514)

The protein subunits of the mammalian cardiac gap junctional hemichannel (a hexamer called a connexon) have recently been extensively characterized. The methods used were protein chemistry,1-3 partial amino acid sequencing,4 and the determination of the complementary DNA sequences corresponding to the entire amino acid sequence of the subunits.5 To follow up these advances, a logical next effort might consist of experiments to elucidate the synthesis of gap junctional channel protein subunits, their assembly and membrane insertion, and the localization of their precursors and breakdown products at various stages of this sequence of events. It seems probable that immunofluorescent and immunoelectron microscopic localizations of antibodies to cytoplasmic surface and other determinants of the channel protein subunits, using antibodies to defined amino acid sequences, will constitute an important tool in the accomplishment of this goal.

A major obstacle to accurate immunoelectron microscopic localization is, however, the rather limited ultrastructural definition of the relevant intracellular and extracellular membrane systems that is achievable with even the best current methods for immunoelectron microscopy. This problem is particularly important because certain structures associated with mammalian cardiac gap junctions (GJs) require unusually good ultrastructural resolution for their detection and identification. The structures in question fall into two categories. The first category consists of gap junction-associated vesicles (GJAVs), which are found extracellularly at the intercalated disks, in the T tubules near the disks, and at selected areas of the cell surface. These vesicles, first described by one of us more than 20 years ago in cat and rat hearts,6,7 were previously shown to be paired by GJ with each other and with adjacent T-tubular plasma membrane. GJAVs have also been found and extensively described in human hearts.8 The second category (described in this paper) consists of an intracellular complex made up of membrane-enclosed GJs and vesicles that are either dissociating from or associating with the GJ.

We have therefore restudied the ultrastructure of cardiac GJs and GJAVs to provide a foundation for future immunoelectron microscopy of these structures. By comparing electron micrographs of conventional, positively stained sections with electron micrographs of hearts negatively stained in situ with lanthanum,9 we have been able to demonstrate that...
some of the GJAVs contain the classical polygonal arrays of connexons characteristic of an assembled gap junctional lattice. The systematic use of in situ negative staining shows that cardiac GJs are subdivided by connexon-free aisles into microdomains. This technique has also revealed that the total membrane area of assembled GJ at the intercalated disks of mammalian heart muscle greatly exceeds previous estimates based on morphometry of thin sections that had been positively stained with uranyl acetate and lead citrate.11–13

Materials and Methods

Preparation of Tissue for Electron Microscopy of Thin Sections

Conventional, positively stained tissues. Sheep atria, dissected from hearts obtained at the slaughterhouse immediately after death of the animal, were immersed for 60–90 minutes in buffered 3% glutaraldehyde with or without 0.5% tannic acid, cut into small cubes <1 mm on a side, rinsed in Na cacodylate buffer, and transported to the laboratory for postfixation in buffered 0.8% OsO4. Female Sprague-Dawley rats weighing 200–300 g were anesthetized with ether and their hearts were excised. The primary fixation of the ventricles was by intravascular perfusion at 22–24°C on the Langendorff cannula as described previously,7,12 using OsO4 with or without 0.5% tannic acid or glutaraldehyde with or without 0.5% tannic acid, all buffered to a final pH of 7.4 with Na cacodylate. Tannic acid was used chiefly to preserve and help to opacify basal lamina. Hearts fixed by primary glutaraldehyde or glutaraldehyde-tannic acid perfusion were cut into cubes 1 mm on a side and postfixed by immersion in ice-cold Na cacodylate-buffered OsO4. The conclusions in this paper are based on the examination of atria from four sheep hearts and ventricles from 23 rat hearts. Blocks of embedded ventricular tissue from a 2-day-old neonatal rabbit used in a previously published study14 were also resectioned for further study.

Several variations in the fixatives were used for perfusion fixation or immersion fixation to demonstrate that the structures of interest (GJs and GJAVs) were present and similar in structure, as observed with different methods of fixation.

The compositions of the three chief primary fixatives for conventional positive staining were as follows: For primary fixation by immersion in glutaraldehyde: 3% glutaraldehyde buffered with Na cacodylate, 150 mM, adjusted to pH 7.4 (followed by postfixation with 32.7 mM OsO4). For primary fixation with perfusion with OsO4 (mM): osmium tetroxide 32.7, NaCl 91, KCl 5.93, CaCl2·2H2O 1.40, MgCl2·6H2O 0.56, and Na cacodylate HCl 50. Some sheep atria were also postfixed (after primary fixation with glutaraldehyde) by a modification of the ferrocyanide-containing solution of Forbes et al.,15 whose composition was 0.8% K2Fe(CN)6·3H2O, 0.8% OsO4 in 150 mM Na cacodylate (pH 7.4). After rinsing free of fixative, tissue blocks were embedded in Epon, thin-sectioned, and stained with uranyl acetate and lead citrate.

In vitro negative staining. For this purpose we perfused rat hearts on the Langendorff cannula for 7 minutes at 37°C with 120 mM Na cacodylate (pH 7.4) containing 1% La(NO3)3 and either 1% glutaraldehyde or 0.8% OsO4. Only the right ventricle was used because preliminary experiments suggested that retention of precipitated lanthanum salts in the GJ by this chamber was superior to that of left ventricle. For experiments with OsO4–La(NO3)3 perfusion, fixation was followed by three 5-minute rinses of 1 mm3 pieces of fixed tissue in ice-cold 75 mM Na cacodylate (pH 7.4) containing 2% La(NO3)3. Dehydration for embedding in Epon was carried out in five stages of 5 minutes each at room temperature without addition of La(NO3)3.

Measurements of widths of defined portions of gap junctional cross sections and of GJs between paired vesicles were made by projecting negative films of the electron micrographs onto the screen of a Nikon Profile Projector GC-2 (Penco Precision, Northfield, Illinois), equipped with digital readout. The final magnification on the screen was ×1.0 × 106.

Morphometry of the fraction of transverse cell boundary at the longitudinal ends of negatively stained right ventricular myocytes was done as described by Stewart and Page,13 with the modification that intersections on one edge of the en face views of the GJ were counted, as well as intersections on the linear profiles of the cross-sectional GJ and on the nonjunctional plasmalemma at the intercalated disk. Only sections in which the longitudinal axis of the cell could be clearly defined (thus defining the mean orientation of the intercalated disk, which is oriented perpendicular to that axis) were used for intersection counts. The procedure of counting one edge of en face GJ areas assumed that, on the average, the widths of junctions cut en face were comparable to those of junctions seen only in the classical five- to seven-layered cross-sectional profiles if the latter were cut en face. Counting was done at a final magnification of ×55 × 103 on junctions traced onto tracing paper from the image of the negative projected onto the surface of a Durst enlarger (model S-45).

Results

In Situ Negative Staining With Lanthanum

Apparent subdivision of connexon arrays. Figures 1–8 are electron micrographs of rat right ventricle
FIGURE 1. Rat right ventricle (fixation and staining method: perfusion with buffered glutaraldehyde+La(NO₃)₃, illustrating in situ negative staining with lanthanum. Left upper half of figure shows elliptical profile of a gap junction cut en face at left and right poles (arrowheads, showing characteristic connexon structure) and cut in cross section elsewhere (star). Lower half shows large area cut in face view. Magnification, ×57.5×10³.

FIGURE 2. Longitudinal thin section of rat right ventricle (fixation and staining method: perfusion with OsO₄+La(NO₃)₃, illustrating that large areas of the intercalated disk consist entirely of face views of the gap junction. These areas are not detectable by conventional uranyl acetate and lead citrate staining. Star shows a doughnut-shaped gap junction profile from the end of a finger-like extension of the intercalated disk whose connection with the doughnut-shaped profile presumably lies above or below the plane of section. Magnification, ×42.5×10³.
opacified with lanthanum by perfusing the hearts with Na cacodylate buffer containing either 0.8% OsO₄+23.1 mM La(NO₃)₃ (Figures 2–7) or 1% glutaraldehyde+23.1 mM La(NO₃)₃ (Figure 1). Although purely negative staining with glutaraldehyde-lanthanum clearly shows the connexon arrays of GJ (Figure 1), the low-contrast positive staining produced by OsO₄ (Figures 2–7) makes the combination of OsO₄ with lanthanum more useful for localizing GJs and GJAVs with respect to other cellular membranes and organelles. [It should be stressed that primary fixation and opacification with OsO₄-La(NO₃)₃ was successful only when these agents were introduced by brief direct intravascular perfusion on the Langendorff cannula, as previously described.⁷] In Figures 2, 3, 4 and 7, the large areas of GJs sectioned en face show characteristic arrays of subunits. A striking feature of these face views is that the junctions appear to be subdivided into microdomains. The connexon-free "aisles" subdividing the junctional surface into microdomains presumably contain precipitated lanthanum oxide or hydroxide. In some projections (e.g., Figure 4), these aisles seem to divide portions of the en face sections of the GJs into roughly polygonal (four- to six-sided) areas (closed microdomains) containing 20 to 39 profiles of cross-sectioned connexons (mean ±SEM 31±2 connexons per polygonal area, n=10 junctions). The arrays of connexons within these...
FIGURE 5. Thinly sectioned rat right ventricle [fixation and staining method: perfusion with OsO₄ + La(NO₃)₃] showing round profiles of vesicles lying in the interstitial space at the intercalated disks (arrow). The profiles appear to contain tightly packed connexons. Magnification, ×42.5×10³.

FIGURE 6. Singly (left) and doubly (right) printed enlargements of vesicular profiles from Figure 5, confirming that the interior of the sectioned profiles is packed with arrays of connexons. Magnification, ×102.5×10³.
areas, though sometimes approaching a hexagonal arrangement, are usually distorted by the nonplanarity of the junction and other factors. Other microdomains are clearly not closed; their limiting aisle does not form a closed structure, so that the enclosed connexon-containing areas are continuous with other incompletely closed microdomains.

**Underestimation of gap junctional area by conventional positive staining.** A second observation, which becomes evident by comparing negatively stained sections prepared by OsO<sub>4</sub>-La perfusion of rat right ventricle (Figures 2 and 4) with sections fixed by OsO<sub>4</sub>- or glutaraldehyde-perfusion+OsO<sub>4</sub> postfixation and subsequently conventionally stained with uranyl acetate and lead citrate (Figures 8-17), is that conventional positive staining of heart muscle for electron microscopy fails to detect most of the gap junctional area present in the section because most of that area is usually present in face views. Figure 8, a portion of an intercalated disk of OsO<sub>4</sub>-La(NO<sub>3</sub>)<sub>3</sub>-fixed ventricle positively stained with uranyl and lead salts, shows the characteristic five- to seven-layered structure of cross-sectioned GJs. The areas cut in face view can be tentatively identified on the basis of experience gained from looking at sections prepared with OsO<sub>4</sub>-La, but such identification is at best provisional. Since morphometry of cardiac GJs has always been carried out by counting only intersections of grid lines with cross-sectioned junctions and not face views,11-13,17-19 we have tried to determine by how much the total gap junctional area at the intercalated disks has been underestimated because of failure to include face views of the junctions. For this purpose, we measured (as described in "Materials and Methods") the percentages of the area at one end of rat right ventricular myocytes contributed by nonjunctional plasma membrane, cross-sectioned GJs, and GJs sectioned in face view in hearts perfused with OsO<sub>4</sub>-La. This experiment indicated that the percentages of a myocyte's transverse cell boundary area at one end of the cell were, respectively, classical cross-sectioned GJs, 6±1; GJs sectioned in face view, 17±3; and nonjunctional plasma membrane 77±9 (n=10 transverse cell boundaries). Assuming that essentially all of the GJ is located within two sarcomere lengths of the intercalated disk, failure to count the junctions sectioned in face view would thus underestimate gap junctional area at the intercalated disks of rat ventricle by 73%; i.e., there was 3.8-fold more gap junctional area than would be estimated on the basis of counting only the pentalaminar profiles of the cross-sectioned junctions in sections conventionally opacified with uranium and lead salts.

**Some gap junction-associated vesicles contain connexons.** A third observation is the presence of vesicular profiles, some of which contain polygonal arrays of connexons in the interstitial spaces at the intercalated disks where the two plasma membranes that form the GJ split into separate membranes with a concomitant widening of the interstitial gap between them (Figures 5-7). These vesicular profiles may be seen lying free in the interstitial space (Figures 5 and 6) or apposed to the extracytoplasmic surface of the plasmalemma bounding that space (Figure 7). The two vesicles in Figure 7 are devoid of aisles between connexons, whereas the nearby en face sectioned GJ in the same figure shows characteristic subdivision by aisles. Vesicular profiles, apparently without detectable subunit structure, are often found interspersed among vesicles containing classical connexon arrays (not shown). In sections stained conventionally with uranyl acetate and lead citrate without impregnation with La(NO<sub>3</sub>)<sub>3</sub>, the corresponding vesicular profiles appear either as single vesicles lying in the interstitial space or as vesicles paired to form classical pentalaminar GJs (Figures 9-13).

Figure 9 shows the pentalaminar configuration of a GJ between two profiles of GJAVs in the lumen of a sheep atrial T tubule,20 fixed with glutaraldehyde-OsO<sub>4</sub>-ferrocyanide and stained with uranium and
lead salts. Figure 10 shows similar vesicular profiles, some paired to form a GJ, lying in the interstitital cleft between two sheep atrial myocytes.

Figures 5-7 suggest that paired vesicular profiles that appear pentalaminar when positively stained with uranyl acetate or with uranyl acetate plus lead citrate contain classical connexons when negatively stained with lanthanum salts and sectioned en face through the junction. The demonstration of connexon arrays in the vesicular profiles was feasible because, by appropriately shortening the duration of rinsing, it was possible to retain lanthanum both in the fully assembled GJ and in the nearby GJAV. Although similar vesicular profiles are present at the cell surface (Figures 11-13), it proved impossible to retain lanthanum deposits in this location because precipitated lanthanum was invariably washed out during the rinsing and dehydration required for embedding.

Characteristics of Extracellular Gap Junction–Associated Vesicles at the Myocyte Cell Surface

Figure 11 shows multiple vesicular profiles lying within the laminar sheath or coat ("basal lamina") of a sheep atrial myocyte where its surface abuts on the wide interstitial space. Both single vesicular profiles and vesicular profiles paired by a GJ-like pentalaminar membrane complex are present. Visualization of the laminar coat has been improved by primary glutaraldehyde fixation, followed by secondary fixation with OsO4 and tannic acid, as well as by the use of semithin (rather than thin) sections. In Figure 12, similar vesicular profiles are seen lying under the laminar coat in an indentation of the surfaces of two rat left ventricular myocytes where their respective plasma membranes approach each other to form a GJ.

The major and minor axes of elliptical profiles of sheep atrial GJAVs lying in laminar sheaths at the free interstitial surface of the myocytes were measured at a final magnification of 100-fold on negatives projected onto the screen of a Nikon Profile Projector. The entire thickness of the positively stained limiting membranes were included in the measured axes. Three types of vesicular profiles were identified and separately measured: Type 1, single vesicular profiles without opaque cores; Type 2, compound vesicular profiles that had paired by forming GJs with each other; and Type 3, single vesicular profiles with opaque cores. The dimensions of Type 1 vesicular profiles (n=50) were (in nanometers, mean±SEM) major axis, 80±4; minor axis, 79±3; calculated circumference, 500±20. For Type 2 (n=35), dimensions were major axis, 88±2; minor axis, 88±3; calculated circumference, 554±21. For Type 3 (n=15), we measured separately both the profile of the outer membrane and the profile of the core. The dimensions for outer membrane were major axis, 84±7; minor axis, 82±6; for the opaque core, major axis, 65±7; minor axis, 62±6. It is apparent that for all three types of vesicular profiles, the major and minor axes were so similar as to suggest that the vesicles were spheres slightly compressed during sectioning. Compound vesicular profiles (Type 2) containing a GJ were clearly larger (2p<0.001) than single vesicular profiles (Type 1).

To compare the dimensions of pentalaminar junctions between profiles of GJAVs and classical cross-
**Figure 9.** Thin-sectioned sheep atrium (fixation and staining method: immersion in glutaraldehyde, postfixation in OsO4+Na ferrocyanide, followed by staining with uranyl acetate and lead citrate). The lumen of an atrial T tubule contains profile of paired vesicles forming a classical pentalaminar profile. Inspection of the figure with ×7 enlarger shows that cytoplasmic surface leaflets have characteristically broadened and dense cytoplasmic surface staining at the junction. Magnification, ×82.5×10^3.

**Figure 10.** Thin section of intercellular cleft between two sheep atrial myocytes, merging at the lower right into a junction between the two cells at the intercalated disk (fixation and staining method: immersion in glutaraldehyde, postfixation in OsO4+Na ferrocyanide, followed by staining with uranyl acetate and lead citrate). The cleft contains profiles of multiple vesicles of various sizes, some of which are joined by gap junction (arrowheads). Note that use of ferrocyanide defines a distinct laminar coat ("basal lamina") covering the extracellular surfaces of the myocyte plasma membranes. Magnification, ×41.3×10^3.
FIGURE 11. Semi-thin section of sheep atrial myocyte (fixation and staining method: immersion in glutaraldehyde + tannic acid, secondary fixation in OsO₄, followed by staining with uranyl acetate and lead citrate). The free surface of the myocyte facing the interstitial space (IS) is covered by four layers of laminar coat that apparently make up two laminar sheaths (large arrows). Vesicular profiles, some of which form pentalaminar junctions with each other (small arrows), lie in these sheaths. Semi-thick sections define junctions between cells less clearly but give better sample of the vesicle population. Magnification, ×41.3×10³.

FIGURE 12. Thin section of rat left ventricle (fixation and staining method: perfusion with OsO₄+tannic acid followed by staining with uranyl acetate and lead citrate) showing vesicular profiles, one with characteristic pentalaminar junction (arrow), lying enclosed by two layers of laminar coat at the free cell surface near the point of formation of a gap junction. Other vesicles are seen in interstitial spaces at the intercalated disk (arrowheads). Magnification, ×64.1×10³.
FIGURE 13. Semi-thin section of sheep atrium (fixation and staining method: immersion in glutaraldehyde+tannic acid, secondary fixation in OsO₄, followed by staining with uranyl acetate and lead citrate) showing a segment of an atrial myocyte cell process lying in the interstitial space. At both ends, the segment of myocyte cell process terminates in sheaths of laminar coat containing multiple vesicular profiles of various sizes (arrows). Magnification, ×15.0×10³. Inset, taken from area near star, shows presence of pentalaminar junctions between paired vesicular profiles. Magnification, ×76.3×10³.

sectioned GJs, we used the Nikon Profile Projector to measure the cross-sectional widths of 18 large GJs in the same sections used for measuring the corresponding widths of GJs from 18 paired profiles of GJAVs from rat left ventricles fixed by perfusion with OsO₄ and stained with uranyl acetate and lead citrate. This comparison of GJ with GJAV yielded the following values (in nanometers, mean±SEM): total width of the junction (cytoplasmic surface to cytoplasmic surface), GJ 30.2±0.9, GJAV 32.0±0.6; total width of the junction minus the width of the two layers of cytoplasmic fuzz, GJ 19.8±0.6, GJAV 20.3±0.3; width of gap, GJ 5.5±0.2, GJAV 5.1±0.1. The cross-sectional dimensions of the GJ and GJAV are therefore very similar, the slight difference being accounted for by the slightly greater thickness of the cytoplasmic surface layer of fuzz in GJAVs.

Profiles of GJAVs were absent from large areas of the free cell surface in both sheep atrium and rat ventricle; that is, regions with GJAVs were localized to areas near large GJs where the transverse cell boundary (intercalated disk) intersected the external sarcolemmal envelope. In such regions of GJAV localization (e.g., Figures 11 and 12), morphometry by intersection counting (as described for plasmalemmal caveolae by Levin and Page²¹) showed that the total area of nonjunctional plasmalemma in the limiting membrane of GJAVs exceeded that of the underlying cell surface plasmalemma by 2:1 to 3:1. Figure 12, prepared by coronary perfusion of a rat left ventricle with buffered OsO₄-tannic acid fixative, illustrates the laminar sheath enclosing vesicles at the free cell surface where the free cell surface intersects the transverse cell boundary. The figure also shows other vesicles within the widened interspaces between plasma membranes of two cells where their transverse cell boundaries confront each other at the intercalated disks.

Intramyocytic Gap Junction-Associated Vesicles

In the course of the above-described studies on GJs and GJAVs, we became interested in certain structural specializations most commonly present in the subplasmalemmal regions of rat and rabbit ventricular myocytes (Figures 14 and 17) but sometimes also observable adjacent to T tubules further inside the cells (Figures 15 and 16). Figure 14 shows subsarcolemmal GJ associated with multiple vesicular profiles of various sizes, some of which appear to be in the process of detaching from (or conceivably forming) small arcs of the gap junctional membrane. Other vesicles lie some distance from the "dissociating" GJ within an elliptical space delimited by a single membrane that is clearly not gap
FIGURE 14. Semi-thin section of rat right ventricle (fixation and staining method: perfusion with OsO4, followed by staining with uranyl acetate and lead citrate) showing an intramyocytic gap junction situated just below the plasmalemma facing the interstitial space. Multiple round and semicircular vesicular profiles appear to be dissociating from (or associating with) gap junction. Additional vesicular profiles lie within a space surrounded by a poorly defined membrane that seems to be continuous with the gap junction. Magnification, x50.0×10³.

To examine the three-dimensional extent of the structures shown in Figures 14–16, we studied them further by serial sections. Figures 17A–F are serial sections of the left ventricle from a 2-day-old (neonatal) rabbit. We have previously shown that at this age there is a sudden, at least fourfold increase in the area of gap junctional membrane per unit myocyte volume.¹⁴ The 2-day-old rabbit heart is therefore useful for investigating ultrastructural changes during a developmental interval when the rates of synthesis and assembly of GJs greatly exceed the rates of their disassembly and degradation. In Figures 17A–F, the GJ is revealed by serial sections to lie inside a cylindrical, membrane-limited structure. The GJ may be tangent to or continuous with the limiting membrane of the cylinder (Figures 17B and 17C), or it may lie free and unattached to the limiting membrane (Figures 17E and 17F). As the series of sections progresses, the GJ becomes subdivided into arcs, each arc presenting its concave surface exteriorly to the cavity and its convex surfaces to the interior of the gap junctional profile. Each concavity gives rise to profiles of various-sized vesicles, some of which come to lie tangent to the membrane limiting the subsarcolemmal space (Figures 17A and 17E). The shape and area of the GJ itself tapers; it seems (in Figure 17E) to be breaking up into vesicular profiles. In the sections shown here, the rim of cytoplasm separating the membrane that delimits the cylindrical cavity from the plasma membrane remains unbroken; that is, no direct continuity between the cavity and the interstitial space was detected.

Discussion

The new findings obtained in this study are 1) the observation that the area of the transverse cell boundary occupied by GJs in mammalian ventricular myocytes is several times greater than previous estimates because of large areas of junction sectioned in the en face orientation and not detected by classical preparative methods; 2) the observation that connexon arrays of thin-sectioned mammalian cardiac GJs in situ, like freeze-fractured GJs¹⁴,²² and isolated cardiac GJs that have been negatively stained with uranyl acetate,² are divided by connexon-free aisles into microdomains; 3) the direct demonstration that a fraction of the profiles of extracellular GJAVs (some of which are paired to form GJs with each other and with myocyte plasma membrane) contain aisle-free arrays of connexons; 4) the demonstration in hearts fixed by appropriate special techniques incorporating tannic acid that GJAVs are present in laminar sheaths ("basal lamina") at the free myocyte cell surface; 5) the demonstration of cylindrical or lamina-shaped membrane-limited compartments, lying either just beneath the plasma membrane of the myocyte or in the interior cytoplasm and usually containing closed gap junctional profiles from which vesicular profiles are seen dissociating into the membrane-
limited compartment (or, alternatively, associating with the GJ from that compartment).

Revision of Previous Estimates of Cardiac GJ Content

The threefold to fourfold underestimation of cardiac GJ area at the intercalated disks, which results from failure to detect the large areas of GJs cut in face view, implies that the number of fully assembled cell-to-cell channels potentially capable of electrically coupling cardiac cells in situ is much larger than previously thought. Inferences about overall junctional conductance and the fraction of total channels that are open, based on correlating measurements of cable properties with morphometric estimates of both GJ membrane area per unit cell volume or per unit heart weight and the number of cell-to-cell channels per unit GJ membrane area,22,23 will have to be revised to take into account these new observations. Measurements on gap junctional membrane area performed on electron micrographs of cardiac Purkinje fibers,18,19,24 a tissue widely used for electrophysiological studies on GJs and other plasmalemmal channels, now need to be reevaluated using La(NO₃)₃ to redefine the total GJ area.

Gap Junction-Associated Vesicles

Since GJs are known to be formed by plasma membrane, we assume that all the vesicles paired by GJs are plasmaleminal vesicles. The evidence that these vesicles contain gap junctional channel protein subunits assembled into connexons is threefold: the direct demonstration of connexon arrays in some of the vesicular profiles after intravascular perfusion with OsO₄-La(NO₃)₃; the observation that the vesicles which have been positively stained are joined by pentalaminar gap junctional complexes with each other or with the membrane of T tubules within two sarcomere lengths of the intercalated disks7; and the observation that the cross-sectional dimensions of profiles of positively stained pentalaminar complexes between paired GJAVs are indistinguishable from those of classical large GJs in the same section. The attribution of a plasmaleminal origin to vesicles associated with GJs but neither paired to form GJs in cross-section nor containing connexon arrays remains unverified, as does the speculation that such unpaired vesicles may contain the channel subunit protein, connexin.

One reason why connexon substructure was demonstrable in only a minority of the profiles of GJAVs negatively stained with La(NO₃)₃ may be that such a demonstration requires that the plane of section pass in the en face orientation through the small (20 nm) thickness of the junction between paired vesicles (not counting the width of the cytoplasmic surface domain), an event with a relatively low probability. By contrast, configurations in which the GJ between paired vesicles are cut in cross section are more frequently observed because the appropriate conjoint orientations of the GJ and knife required are much less restrictive than those needed to obtain en face views of the connexon arrays between two vesicles.

Profiles of GJAVs are present both after glutaraldehyde fixation and in hearts fixed by direct perfusion with OsO₄ without exposure to glutaraldehyde (Figure 12 and Meddoff and Page7). Nevertheless, it is conceivable that these vesicles arise by fragmentation of GJ as an artifact of chemical fixation. Similarly, we cannot rule out that some apparently independent vesicular profiles are created by sectioning a highly convoluted portion of a twisting gap junctional surface.
FIGURE 17. Semi-thin consecutive serial sections of left ventricle from 2-day-old neonatal rabbit (fixation with OsO₄). See text for discussion. Magnification, ×57.5×10⁴.
Extracellular Location of Gap Junction-Associated Vesicles

The functions (if any) of extracellular GJAV are at present unknown. If the vesicles are independent structures, they represent extracellular depots of gap junctional channel proteins, some of them assembled into junctional channel proteins, some of them assembled into lysosomal membrane and that the complexes represent the intramyocytic complexes of Figures 17A-F is a priori grounds that, like GJ from liver, cardiac GJ is that these structures are associated with plasma membranes of neighboring cells might reasonably be expected to occur. We have recently found that extracellular vesicles paired by GJ are also present in primary cultures of rat atrial myocytes. In these cultures, prepared as described by Iida et al., such paired vesicles first appear abruptly on the fourth day of culture at a time when cell processes of atrial myocytes form GJs with each other and when the first coordinated contractions involving multiple myocytes become evident.

Intramyocytic Complexes of Gap Junctions and Vesicles

The most probable interpretation of the intramyocytic complexes (consisting of membrane-enclosed GJs, vesicles, and vesicles apparently dissociating from GJ) is that these structures are associated with turnover of gap junctional channels. It is clear on a priori grounds that, like GJ from liver, cardiac GJ channels must turn over, although the turnover rate of the cardiac GJ channel protein remains to be determined. In this regard, it is possible that the cylindrical or cigar-shaped membrane surrounding the intramyocytic complexes of Figures 17A–F is a lysosomal membrane and that the complexes represent a step in lysosomal degradation of GJ.

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