Original Contributions

Fate of Gap Junctions in Isolated Adult Mammalian Cardiomyocytes

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The fate of gap junctions in dissociated adult myocytes, maintained for up to 22 hours in culture medium, was investigated by semiquantitative analysis of thin sections and by freeze-fracture electron microscopy. Gap junctions in the dissociated myocyte are intact bimembranous structures seen either as invaginated surface-located structures or as annular profiles in the cytoplasm. Surface-located junctions are sealed from the exterior by a sheet of nonjunctional membrane originating (together with the "outer" junctional membrane) from the formerly neighboring cell. Serial sectioning was used to establish that at least part of the annular gap junction population in the freshly isolated myocyte represents truly discrete cytoplasmic vesicles; thus, some gap junctions are rapidly endocytosed after myocyte separation. Analysis of the surface-located–to–annular gap junction ratio suggested that no further endocytosis occurred in rabbit and cat myocytes maintained for 22 and 15 hours, respectively. Guinea pig myocytes, by contrast, did appear to continue endocytosis in culture. Analysis of the distance of gap junctional structures from the cell surface suggested that little if any inward migration of gap junction vesicles occurred. Hypoxia had no detectable effect on the internalization or inward movement of gap junctions. The quantity of ultrastructurally detectable gap junction membrane appeared to remain constant over time, as did the incidence of "complex structures" (i.e., annular gap junction profiles with features previously suggested to represent degradation). New gap junction formation was negligible, and a reappraisal of the nature of "complex structures" led to the conclusion that the origin of these structures need not be related to degradation. Taken together, the findings suggest that degradation and disappearance of gap junctional membrane after isolation of the mature myocyte constitute a much slower process than previously believed, and the possibility that the cardiac gap junction protein has a longer half-life than its counterpart in liver remains open. (*Circulation Research* **1989;65:22–42**)

Gap junctions are specialized plasma membrane domains made up of aggregates of channels that allow the direct exchange of ions and small metabolites between adjoining cells.[1–3] In cardiac muscle, a primary function of the gap junction lies in the electrical coupling of myocytes, enabling coordination of the contractile behavior of the myocardium as a whole.[4,5] Although the gap junctions of cardiac muscle cells reveal many features in common with those of nonexcitable cells, this difference of functional emphasis appears to be reflected in distinct differences in their structure, biochemistry, and composition.[5–7]

Refinements in techniques for the dissociation and culture of myocytes from the mature mammalian heart[8–11] have opened new opportunities for exploration of the specific properties of cardiac gap junctions. Isolated pairs of myocytes have already been exploited in electrophysiological and dye transfer studies[12–14]; other potential applications include use of dissociated and cultured myocytes for 1) the study of cardiac gap junction degradation and formation, and 2) as a starting material for the isolation of pure cardiac myocyte gap junctions from which contamination with junctions of other cell types in the heart (e.g., endothelial cells, smooth muscle cells, and myofibroblasts) can be excluded. Pursuit of these possibilities and definition of the differences between the isolated myocyte and multicellular experimental systems require a detailed understanding of the fate of gap junctions in dissociated and cultured myocytes.

Descriptive electron microscopic studies documenting the initial events associated with myocyte

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separation and the appearance of junctions in freshly isolated cells show that gap junctions often remain as intact bimembranous structures with one of the two myocytes that originally shared the junctions,[15–22] although the possibility of these structures splitting into their component halves has also been considered.[17–19] Annular profiles of gap junction membrane (i.e., apparent cytoplasmic vesicles) are common in the cytoplasm of freshly isolated myocytes.[20,21] One study suggested that after cell dissociation, surface-located gap junctions are progressively internalized over a period of several hours, forming cytoplasmic vesicles that migrate into the cell interior and undergo lysosomal degradation.[22] Other studies on cultured myocytes have reported the disappearance of gap junctions after 12 or 24 hours.[10,23] Gap junctional contact between dissociated myocytes is known to be reestablished in long-term cultures (e.g., 4–28 days[10,24,25]), and has been reported to occur as early as 4 hours[10,26] or after overnight[13] in culture.

In the present paper we explore in detail, by freeze-fracture electron microscopy and by semiquantitative analysis of thin sections, the fate of gap junctions in myocytes isolated from several mammalian species and maintained in culture medium for periods up to 22 hours. In particular, the study set out to examine 1) the extent, if any, to which gap junctions are internalized at the time of cell separation and/or during subsequent maintenance in culture; 2) the possibility that if cytoplasmic gap junction vesicles are formed, they migrate into the cell interior and, if so, how far; and 3) the ultrastructural evidence for the degradation or reformation of gap junctions.

Materials and Methods

Isolation and Culture of Rat, Rabbit, Cat, and Guinea Pig Myocytes

Hearts were obtained from Sprague-Dawley rats (250–300 g), New Zealand White rabbits (1–2 kg), cats (2–2.5 kg), and albino guinea pigs (300–500 g). The animals were maintained on standard laboratory diets and tap water, available ad libitum. Suspensions of purified dissociated cardiac myocytes were prepared by retrograde perfusion of the hearts, via the aorta, with crude collagenase in low-calcium Krebs-Ringer bicarbonate buffer, as described in detail by Powell et al.[27,28] After isolation and purification, the cells were suspended in culture medium in sterile 20-ml tubes at room temperature for periods of up to 22 hours (and in one case, for 40 hours). The culture medium used was Dulbecco's modified Eagle medium (MEM) in 25 mM HEPES containing sodium pyruvate (1 g dm^{-3}), glucose (1 g dm^{-3}), and horse serum (5% vol/vol, mycoplasma-screened). The viability of the cells was ascertained by standard criteria (trypan blue dye exclusion, "rod-shaped" morphology as seen by light microscopy, ultrastructural features, and metabolic and electrophysiological properties).[9,11,20,27–31]

Experimentally Induced Hypoxia

For experiments on hypoxia, cells were incubated at room temperature in sealed conical flasks in a solution containing mannitol (1.8 g dm^{-3}) in 5 mM HEPES plus $MgSO_4$ (1.2 mM), KH_2PO_4 (1 mM), KCl (4.8 mM), and NaCl (118 mM). Before the solution was added to the cells, it was gassed with 100% nitrogen and the air in the flask was displaced with nitrogen before the flask was sealed. Aerobic controls were incubated in a corresponding solution that contained glucose (1.8 g dm^{-3}) in place of mannitol; 100% oxygen was used in place of nitrogen.

Electron Microscopy

For each heart, a sample of dissociated cells was taken for fixation immediately after isolation, and further samples were fixed after various periods of maintenance in culture medium. A similar strategy was adopted for the hypoxia experiments. Fixation was carried out by addition of 1 volume of myocyte suspension to 4 volumes of 2.5% glutaraldehyde in Krebs' or sodium cacodylate buffer (pH 7.3). Glutaraldehyde fixation was continued for 2 hours and followed by rinses in 0.1 M sodium cacodylate. Samples for thin sectioning were routinely postfixed in cacodylate-buffered 1% OsO_4 for 2 hours at 4° C. Some samples were also treated with 0.4% ruthenium red (incorporated in the OsO_4 solution); in these cases the postfixation period was extended to 16 hours. For ease of handling in subsequent processing steps, the postfixed cells were centrifuged briefly in 18% bovine serum albumin to which an equal volume of glutaraldehyde had been added immediately beforehand. Blocks of pelleted myocytes, embedded in the cross-linked bovine serum albumin gel, were stained with uranyl acetate (saturated, in 50% ethanol) for 1 hour at room temperature, dehydrated in ethanol, and embedded in Araldite or Epon via propylene oxide. Ultrathin sections were further stained with uranyl acetate and lead citrate before examination.

In addition to dissociated cells, myocytes of intact tissue were examined as controls. Whole hearts were fixed by retrograde perfusion, via the aorta, with glutaraldehyde fixative, and processed in a corresponding manner to the dissociated cells, as previously described.[32]

For freeze-fracture, buffer-rinsed glutaraldehyde-fixed samples (dissociated myocytes and intact tissue) were infiltrated with cacodylate-buffered 25% glycerol over a period of 1–1.5 hours, mounted, and frozen by immersion in liquid nitrogen–cooled Freon 22 or propane. Freeze-fracturing was carried out either by microtome or by use of a double-replica device in a BAF 400T apparatus (Balzers High Vacuum Ltd, Berkhamsted, England) at −115° C and $2–5\times10^{-7}$ mbar. Standard platinum-carbon rep-

licas were prepared and cleaned in chromic acid or domestic bleach.

Thin sections and freeze-fracture replicas were examined and images recorded with a Model 301 electron microscope (Philips Scientific and Analytical Equipment, Eindhoven, The Netherlands).

Morphometric Analysis of Gap Junctions

After preliminary qualitative examination of dissociated myocytes maintained for a range of intervals between 0 and 24 hours, a more detailed quantitative study on gap junctions was designed as follows. For the rabbit, preparations of cells derived from five different hearts were each divided into two batches, one of which was fixed immediately (0 hour) and the other maintained in culture medium for 22 hours. Images of cells sectioned to give a complete unobstructed two-dimensional view across the entire cell and in which at least one gap junction was visible were photographed for analysis. Twenty such sectional views from each of the 10 batches of rabbit myocytes were used, giving 100 sectional views, each of a different cell, for each time period. A similar procedure was followed with the other species. For the guinea pig, 40 sections of cells were analyzed at 0 hours, and a further 40 sections of cells were analyzed at 5 hours (derived from four hearts; 10 sectional views of cells from each heart at each time point). For the cat, a total of 40 sectional views of cells (derived from two hearts; 10 sectional views per heart per time point) were analyzed. In one experiment on cat cells, the incubation period was extended to 40 hours.

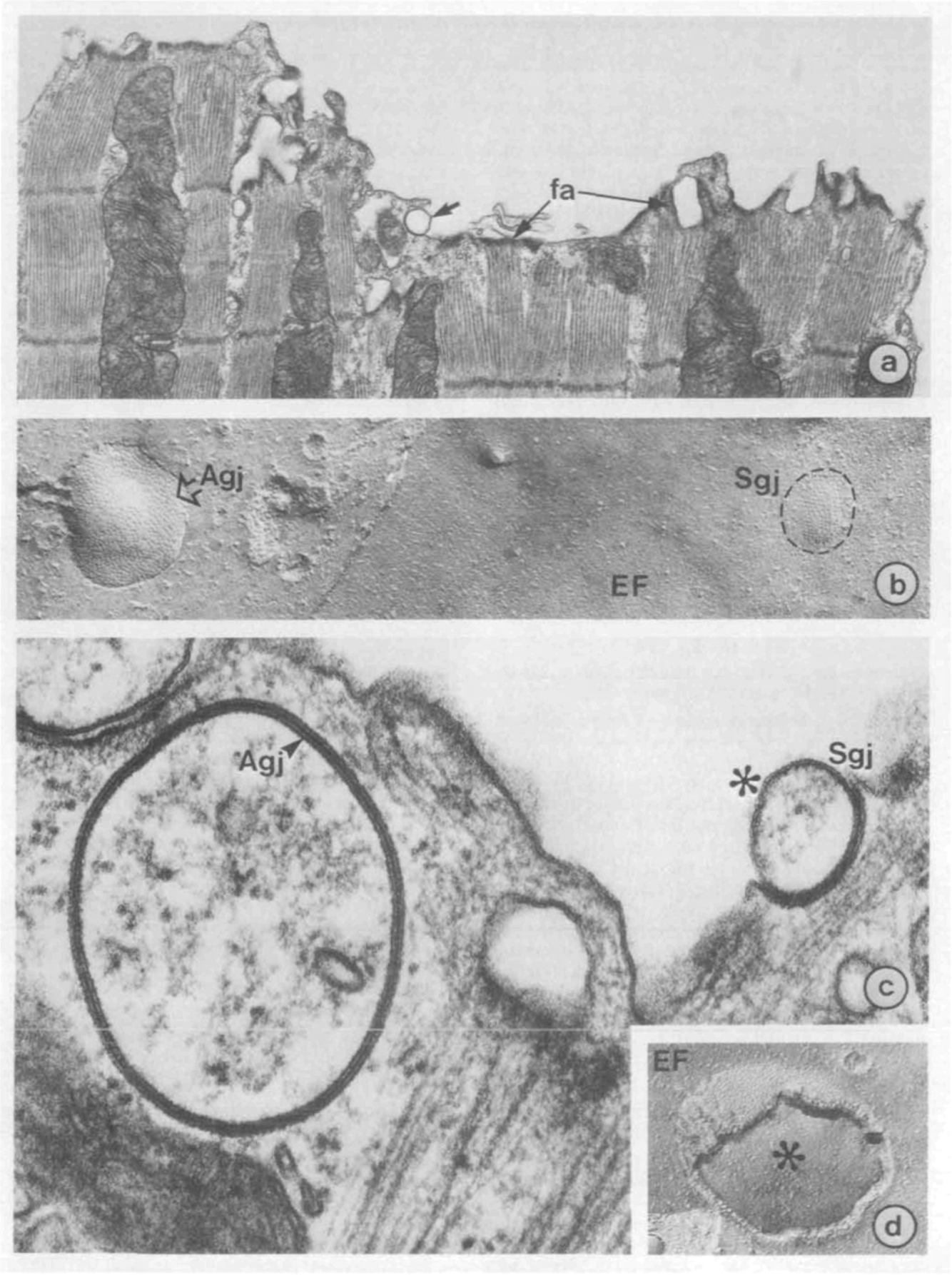
In the hypoxia experiments, myocyte preparations from each of four rat hearts were divided into eight samples for incubations of 0, 30, 60, and 90 minutes with corresponding aerobic controls. Examination by phase contrast light microscopy showed that the 30-minute samples were most suitable for analysis (see "Results," Effect of Hypoxia); 10 sectional views of different cells from each heart were then selected, giving 40 sections of cells for analysis of 30-minute hypoxia and 40 sections for the 30-minute aerobic control.

Micrographs were taken at a magnification of ×18,000 and printed at ×45,000. In each set of sectioned cells, the gap junctions observed were classified into two groups, surface-located (i.e., in visible continuity with the surface plasma membrane) and annular profiles (i.e., apparent vesicles), and the numbers in each category were analyzed. Measurements of the distances of all gap junctional structures from the nearest visible plasma membrane surface (i.e., cytoplasmic surface to cytoplasmic surface) were made, and the sets of data obtained from different hearts of the same species at a given time period were compared statistically with one another by means of the Kruskal-Wallace test. Where no significant difference ($p>0.05$) was found, the data for that time period were pooled. The pooled data for each of the different time periods in a given species were then compared against each other by means of the Wilcoxon-Mann-Whitney U test. For visual comparison of these data, histograms of the frequency distributions of the gap junction distances were plotted. For statistical comparisons of other measurements, standard t tests and χ^2 tests were applied.

Comparisons of the size of apparent gap junction vesicles were made between samples from the time points. The longer diameter of randomly selected annular profiles was first taken, and then the maximum diameter normal to it. The majority of annular gap junction profiles were spheroid or ovoid; those that deviated substantially from this shape were excluded. In addition, as a more sensitive measure of the amount of gap junction membrane present, the profile lengths of all junctional structures were recorded using a VIDS III program (Analytical Measuring Systems, Cambridge, England) with an Olivetti M24 computer (Olivetti S.p.A., Ivrea, Italy) linked to a digitizer tablet. Comparative estimates of gap junction number over time were made by a count of the number of gap junctions visible per cell section in the set of micrographs obtained for each time period. In addition, the relative numbers of cells in the original sections that revealed no gap junctions versus those that revealed one or more gap junctions were determined directly at the electron microscope.

As a separate exercise, a further quantitative analysis was carried out by use of different sets of randomly sectioned cells to establish whether 1) cell volume or surface area, 2) gap junction profile length, 3) gap junction number per sectioned cell, and 4) gap junction surface area per unit cell volume altered significantly during the culture period. For comparison of relative cell volumes between time points, randomly sectioned cells were sampled by recording images at low magnification (×1,300) on the electron microscope. The negatives were enlarged threefold by photographic projection and the cell outlines traced. The sectional areas and the lengths of the tracings were then measured with the digitizer tablet and VIDS III software. The gap junction profile lengths were determined as part of the same analysis. During the low-magnification

FIGURE 1. *Ultrastructure of intercalated disk and gap junctions in freshly isolated rat myocytes.* Panel a: *Survey thin-section view of intercalated disk. Plasma membrane-associated electron-dense zones at myofibril ends are fasciae adherentes (fa) that have been split in half. Arrow indicates a surface-located gap junction.* Panels b *and* c: *Freeze-fracture and thin-section comparison of details of annular gap junction profiles (Agj) and surface-located gap junctions (Sgj). Note loop of sealing membrane (*) in Sgj of panel c, revealed as a membrane sheet in the freeze-fracture view in* panel d. *EF, E-face view of plasma membrane. Magnification: a, ×21,000; b, ×92,400; c, ×122,400; d, ×78,400.*



photography of sectioned cells, each cell was coded and inspected at high magnification for gap junctions. Where gap junction membrane was present in the section plane, it was photographed at ×43,000, and the corresponding cell code was recorded to allow correlation of the low-magnification and high-magnification images. Again, profiles of gap junctional membrane were traced from ×3 photographically projected images of the negatives, and their lengths were measured using the VIDS III system. From these data, the mean profile length of gap junctions, the mean number of gap junctions per cell section, and the mean profile length of gap junction membrane per unit cell area and per unit cell surface were calculated.

The percentage of "complex structures" (i.e., annular gap junction profiles showing complex features previously suggested to represent degradative change[22]) was determined at each time point. For this analysis, our definition of "complex structures" set out to encompass gap junction–related secondary lysosomes, annular junction profiles containing or surrounded by membrane (whether overtly junctional or otherwise), and annular profiles in which a separation of the junctional membranes had occurred; however, it did not include apparent gap junction vesicles that contained mitochondria since the genesis of such structures was manifestly unconnected with degradation.

Results

Ultrastructure of Gap Junctions and Intercalated Disks in Freshly Isolated Myocytes

The intercalated disk regions of freshly isolated, rod-shaped myocytes show well-preserved ultrastructural features, including an intact plasma membrane (Figure 1a). As established previously, dissociation of myocytes involves separation of the two membranes comprising the intercalated disk such that the adherentes junctions are split in half while the gap junctions remain in toto with one or other of the formerly adjacent cells.[15–22] Gap junctional membrane in the dissociated myocyte is seen in two forms: 1) at the cell surface, in visible continuity with the plasma membrane, and 2) as apparent vesicles (i.e., annular profiles) within the cytoplasm (Figures 1b and 1c). Both surface-located and annular gap junctions show the characteristic features of gap junction membrane, that is, a pentalaminar or septilaminar structure in transverse thin section (Figure 1c) and connexon arrays (or their complementary pits) in freeze-fracture (Figures 1b and 2a through 2c).

Surface-located gap junctions. Surface-located gap junctions occasionally lie flat within the plasma membrane (Figure 2d), although most are invaginated to a greater or lesser degree (Figures 1c and 2e). Their innermost membrane is continuous with the neighboring disk plasma membrane of the cell in which they occur. The outermost junctional membrane, derived from the formerly adjacent myocyte, often connects to a loop of nonjunctional membrane (Figure 1c), seen as a continuous membrane sheet in freeze-fracture (Figure 1d). Such junctions are evidently not torn out at the border of the junctional plaque but rather with an attached margin of nonjunctional membrane, the free end of which is capable of fusing together, sealing off the junction from the exterior.

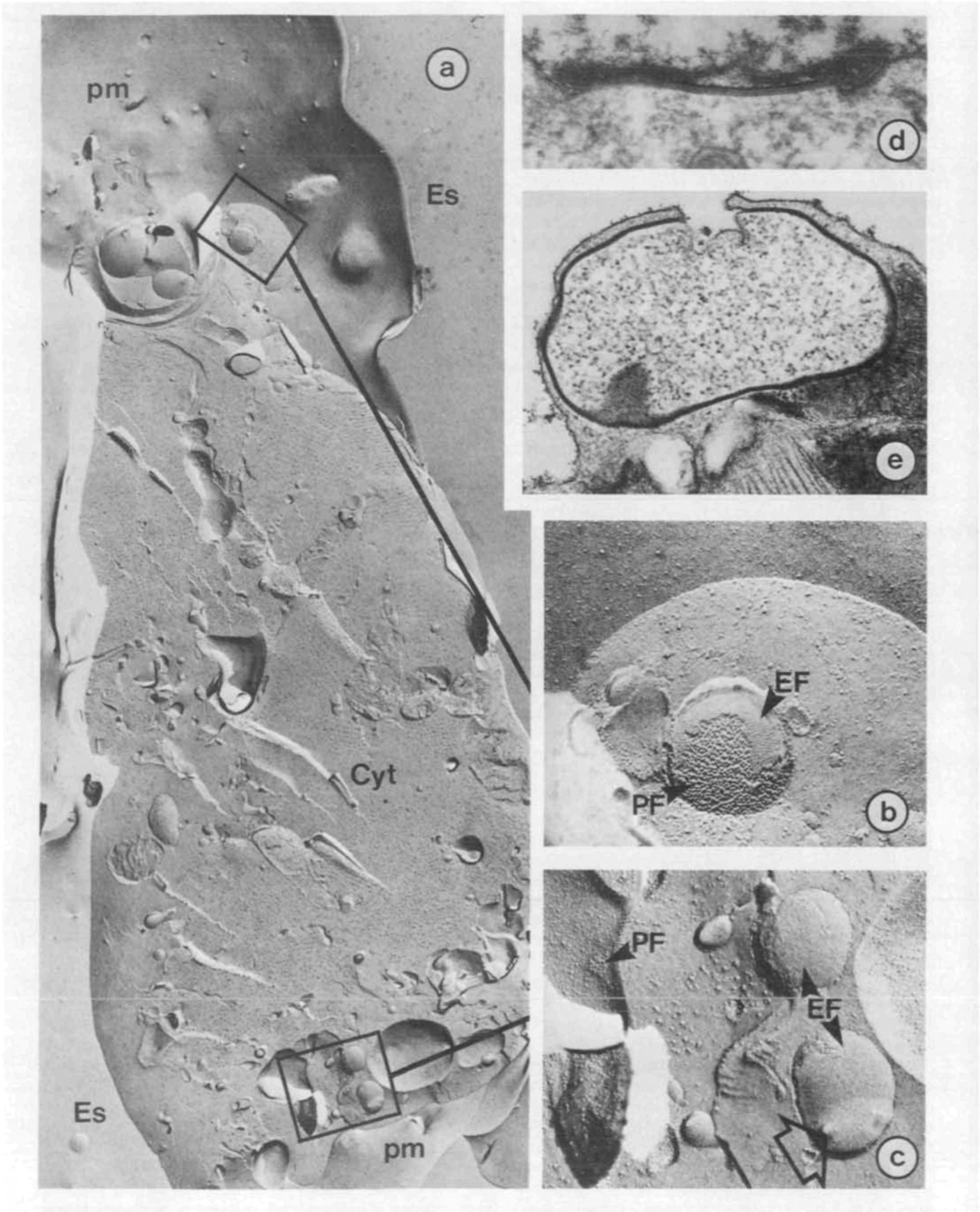
Annular gap junctions. Annular profiles of gap junction membrane can be seen in myocytes of the intact heart (Figure 3a), but their presence is exceptional, accounting for less than one in 300 gap junctions viewed in random sections in all species examined. In the isolated myocyte, by contrast, annular profiles account for 50–75% of the gap junction population.

Depending on the plane of section, deeply invaginated surface-located gap junctions will be viewed on occasion as apparent vesicles within the cytoplasm. However, serial sectioning (Figures 3b through 3g) demonstrates conclusively that a significant proportion of the annular profiles seen in freshly isolated myocytes are truly discrete vesicles that have been detached from the plasma membrane. The serial sections further show that some of the vesicles consist entirely of gap junction membrane. However, a cell that internalizes a surface-located junction that is sealed over with nonjunctional membrane has to pinch off a corresponding portion of its own nonjunctional membrane during endocytosis; thus, the limiting membranes of many gap junction vesicles include varying amounts of nonjunctional membrane (see "Results," "Ultrastructure of complex structures").

Ultrastructure of Myocytes Maintained in Culture Medium

The majority of myocytes maintained in culture medium for up to 22 hours under our experimental

FIGURE 2. Panel a: *Survey freeze-fracture view of intercalated disk region of isolated rabbit myocyte (22-hour sample). The fracture has "cut" transversely across the cell, revealing cytoplasmic gap junction vesicles on either side (framed areas).* Panels b *and* c: *Enlargements of framed areas in panel a. Note characteristic particles on P-face views (PF) of gap junction membrane and corresponding pits on E-face views (EF). Lower gap junction vesicle in panel c is composed partly of nonjunctional membrane (open arrow).* Panels d *and* e: *Thin-section (ruthenium red–stained) views of surface-located gap junctions illustrating variation in degree of curvature from essentially flat (panel d) to deeply invaginated (panel e). Cyt, cytoplasm; pm, plasma membrane; Es, extracellular space. Magnification: a, ×17,700; b, ×59,400; c, ×66,000; d, ×68,800; e, ×46,800.*



conditions retained a "rod-shaped" morphology with well-organized myofibrils and other ultrastructural features typical of the freshly isolated cell[20] (Figures 4a and 4b). However, all the cells maintained for the longer periods showed some degree of smoothing over of the formerly irregular contours of the intercalated disk, as reported after varying periods of culture in other experimental systems.[10] This smoothing-over process initially involves the replacement of the sharply undulating vertical disk segments with a smoothly contoured membrane lying beyond the apparently internalized fasciae adherentes (Figure 4c). Any myofilament disorganization is limited to the ends of the myofibrils, and a clear myofibril-free area of cytoplasm containing annular gap junction profiles and mitochondria often lies just beneath the smoothed plasma membrane. The gross steplike appearance of the disk, comprising alternating vertical and horizontal membrane segments, was retained in most cells, although in some cells the smoothing process had progressed to a stage in which an entire cell end appeared rounded (Figure 4b). Zones of newly established contact between the lateral surfaces of cultured myocytes were detected infrequently (Figure 4d). Apart from occasional punctate appositions, the distance between the plasma membranes participating in these contact zones exceeded that diagnostic of the gap junction. This indicated that extensive formation of new gap junctions had not occurred, although the occasional presence of very small forming gap junctions cannot be excluded. Large surface-located gap junctions (with sealed loops of membrane) and annular profiles lying just beneath the plasma membrane were common, however, as in freshly isolated cells (Figures 4e through 4g). Freeze-fracture showed no loosening of connexon packing and no evidence for connexon dispersal in the membrane plane (Figure 4g).

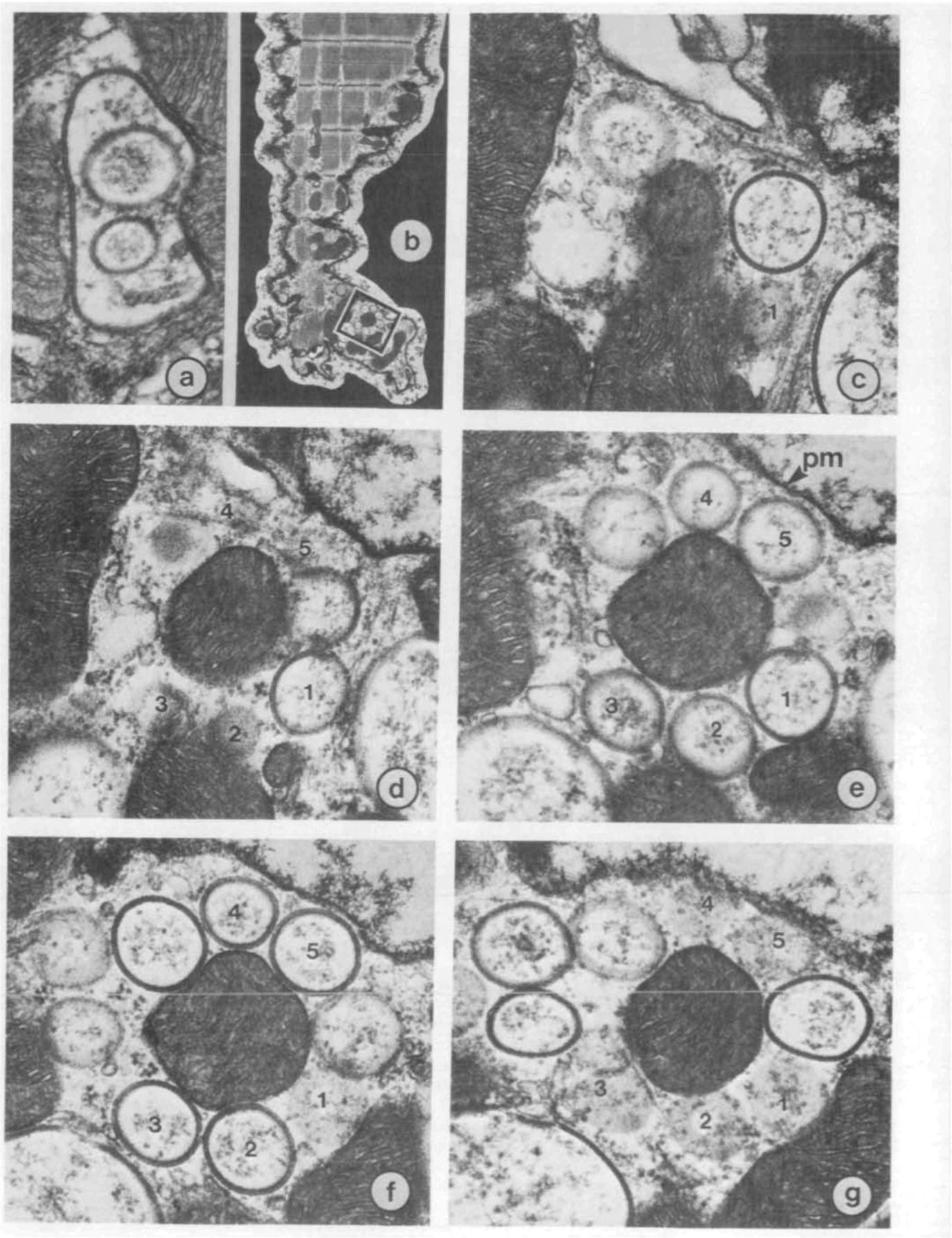
Is Gap Junction Membrane Progressively Internalized by Endocytosis After Myocyte Isolation?

To investigate whether the invaginated surface-located gap junctions visible in freshly isolated cells are internalized during culture to form cytoplasmic gap junction vesicles that subsequently migrate into the cell interior, we measured at time intervals after dissociation 1) the relative numbers of surface-located and annular junctions and 2) the distances of the junctions from the nearest visible plasma membrane. Even though not all annular profiles of gap junction membrane are necessarily truly discrete vesicles, internalization would nevertheless alter the surface-located–to–annular gap junction ratio. Similarly, measurement of the distance of an annular profile from the nearest visible cell surface does not necessarily give its true depth in the cell, but inward vesicle movement would nevertheless result in an overall increase in these measured distances.

Surface-located–to–annular gap junction ratio. Table 1 compares the relative numbers of surface-located versus annular gap junctions immediately after dissociation and after various incubation periods in rabbit, cat, and guinea pig myocytes. In rabbit and cat myocytes maintained in culture for 22 hours and 15 hours respectively, a slight increase in annular profiles at the expense of surface-located junctions was observed, but this alteration was not statistically significant ($p>0.05$), and abundant junctions continuous with or close to the surface were consistently seen (Figures 4e through 4g). In the guinea pig, however, a pronounced and statistically significant ($p<0.01$) diminution in surface-located junctions (with a corresponding rise in annular profiles) occurred over an incubation period of just 5 hours. These results suggest the possibility that the configuration of gap junction membrane in the rabbit and cat myocytes remains stable over prolonged periods, with little if any endocytosis, but that the guinea pig myocytes actively internalize in their gap junctions over a much shorter period.

Gap junction distance from the cell surface. The frequency distributions of the observed distances of gap junctions from the cell surface in rabbit myocytes at 0 and 22 hours are shown in Figure 5. The distances measured for each of the five populations of rabbit myocytes (each from a different heart) analyzed at 0 hours were not significantly different from one another ($p>0.05$); corresponding analysis of the five matched 22-hour samples gave the same result. Accordingly, the 0-hour data were pooled as one group and the 22-hour data as another, and

FIGURE 3. Panel a: *Annular profile of gap junction membrane from intact rat heart. Such profiles are rare in undissociated cells, and where they do occur, they are usually due to convolutions of gap junction membrane that have been sectioned across.* Panel b: *Survey view of isolated rat myocyte (ruthenium red–stained) selected for serial section analysis of annular gap junction profiles.* Panels c *through* g: *Set of serial sections from framed area in panel b. In panel c, annular profile 1 can just be seen as a gray discoid "ghost" representing tangentially sectioned edge of a junctional membrane sphere. Annular profiles 2 and 3, not visible in panel c, first come into view in the same way in panel d. These three gap junctional structures can be traced through panels e and f to their disappearance (in tangential view) in panel g. At no stage in this sequence do they lie adjacent to plasma membrane (pm), and cytoplasmic components are seen in a position corresponding to that of gap junctional structures, at beginning and end of series. Annular gap junction profiles 1 to 3 are thus true cytoplasmic vesicles that have been detached from the plasma membrane. Annular junction profiles 4 and 5 can be traced similarly, and though situated close to the plasma membrane, also appear discrete. Magnification: a, ×55,000; b, ×3,900; c through g, ×52,900.*



statistical comparison between these two sets of data showed no significant difference ($p>0.05$). Similar results were obtained following the same procedure with matched samples of cat myocytes incubated for 0 and 15 hours; moreover, no obvious alteration was observed when the culture period was extended to 40 hours (Figure 6).

In the guinea pig, the distance data for the four 0-hour samples of myocytes analyzed did not differ significantly from one another ($p>0.05$); similarly, no significant difference was observed between each of the corresponding 5-hour samples. However, the pooled 5-hour distances were found to differ significantly from the pooled 0-hour measurements ($p<0.05$). This difference is reflected as a slight reduction in the percentage of those annular profiles that lie closest to the surface and a slight increase in those observed at greater distances in the 5-hour histogram (Figure 7).

In line with the apparent differences in gap junction endocytotic activity, therefore, inward penetration of gap junction vesicles apparently did not occur in the rabbit and cat myocytes but did occur in the guinea pig, albeit to a limited extent.

Effects of cell volume and other factors. The tentative conclusions suggested from the surface-located–to–annular gap junction ratio and gap junction distance from the cell surface in the sections above depend on a number of assumptions. For example, if the volume of the cell alters upon culture, then the distance of a cytoplasmic gap junction vesicle from the cell surface could also change without any real movement of the vesicle. Although altered cell volume per se would not affect surface-located–to–annular gap junction ratio, such an alteration would affect the frequency of section planes revealing gap junctions. The possibility that cytoplasmic gap junction vesicles may undergo fission or fusion or may re-fuse with the plasma membrane, on the other hand, would have a direct effect on the numbers counted in each junction category.

In practice, there was no evidence for an alteration of cell volume (as measured from randomly sampled sectional areas of cells); these data are given in the section of the "Results" that examines the question of gap junction degradation (see subsection entitled "Amount of gap junction membrane: Gap junction profile length"). Also contained in this subsection are results on the mean profile lengths of gap junctions and the mean numbers of gap junctions per random cell section, parameters that also appeared unchanged during the culture period. Fusion of gap junction vesicles would be expected to result in fewer and larger gap junctions, while fission would result in the reverse. The data show no evidence for either trend, though it should be noted that occasional "dumbell" annular profiles, suggestive of fusion/fission, were observed (e.g., Figure 11f). If fusion/fission events do occur, they appear to be rare, or the rates of fusion and fission might conceivably be approximately in equilibrium. We cannot exclude the possibility that some distortion to the ratio estimates may arise from this source, though this would not appear to be a major source of error.

Effect of Hypoxia

To examine whether gap junction internalization might be stimulated by hypoxia, we first established the gross response of isolated myocytes to different periods of hypoxic incubation. In our experimental system, >60% of the original "rod-shaped" population underwent hypercontracture with consequent rounding up and gross damage after 90 minutes of hypoxia. (Figure 8). The myocytes proceeded to this state in stages (Figure 9), the first of which involved an abrupt 33% reduction in sarcomere length (Figure 9b) to form a temporarily stable "shortened rod."[33] After 30 minutes' hypoxic incubation, the majority of myocytes fell into this category, although, because of differences in the rate of response of individual cells, "rods" of normal sarcomere length (Figure 9a) as well as cells that had progressed to hypercontracture (Figures 9c and 9d) were also present. By carrying out a gap junction analysis at the 30-minute stage, however, we were able to work with a cell population that was predominantly free of gross damage but, nevertheless, had shown a clear response to hypoxia.

FIGURE 4. Panels a *and* b: *Survey views of longitudinally thin-sectioned rabbit myocytes maintained in culture medium for 22 hours. The cells have remained rod-shaped, with well-organized, longitudinally oriented myofibrils and interspersed rows of mitochondria. Irregular steplike configuration of intercalated disk regions at ends of cell are still prominent in the myocyte in panel a, but are more rounded as a result of smoothing-over in the myocyte in panel b, especially at right-hand end.* Panel c: *Detail of smoothed-over zone in 22-hour rabbit myocyte. Most of fasciae adherentes region (fa) has retained its plicate configuration despite internalization within the cell (compare Figure 1a). A myofibril-free zone beneath the new, smoothly contoured plasma membrane (spm) contains several annular gap junction profiles (arrowheads). Some myofilament disorganization is evident close to the smoothed zone (open arrows).* Panel d: *Reassociation between two rabbit myocytes maintained in culture for 22 hours. Newly established zone of contact between these cells is filled with a dense amorphous material.* Panels e *and* f: *Thin-section views of annular gap junction profiles adjacent to plasma membrane and surface-located gap junctions (arrows) in rabbit (panel e) and cat (panel f) myocytes maintained for 22 and 15 hours, respectively.* Panel g: *22-hour rabbit myocyte, freeze-fracture view (E-face) of plasma membrane demonstrating that surface-located gap junctions (open arrows) remain readily detectable. Magnification: a, ×2,550; b, ×2,740; c, ×12,900; d, ×5,600; e, ×46,800; f, ×45,000; g, ×70,000.*

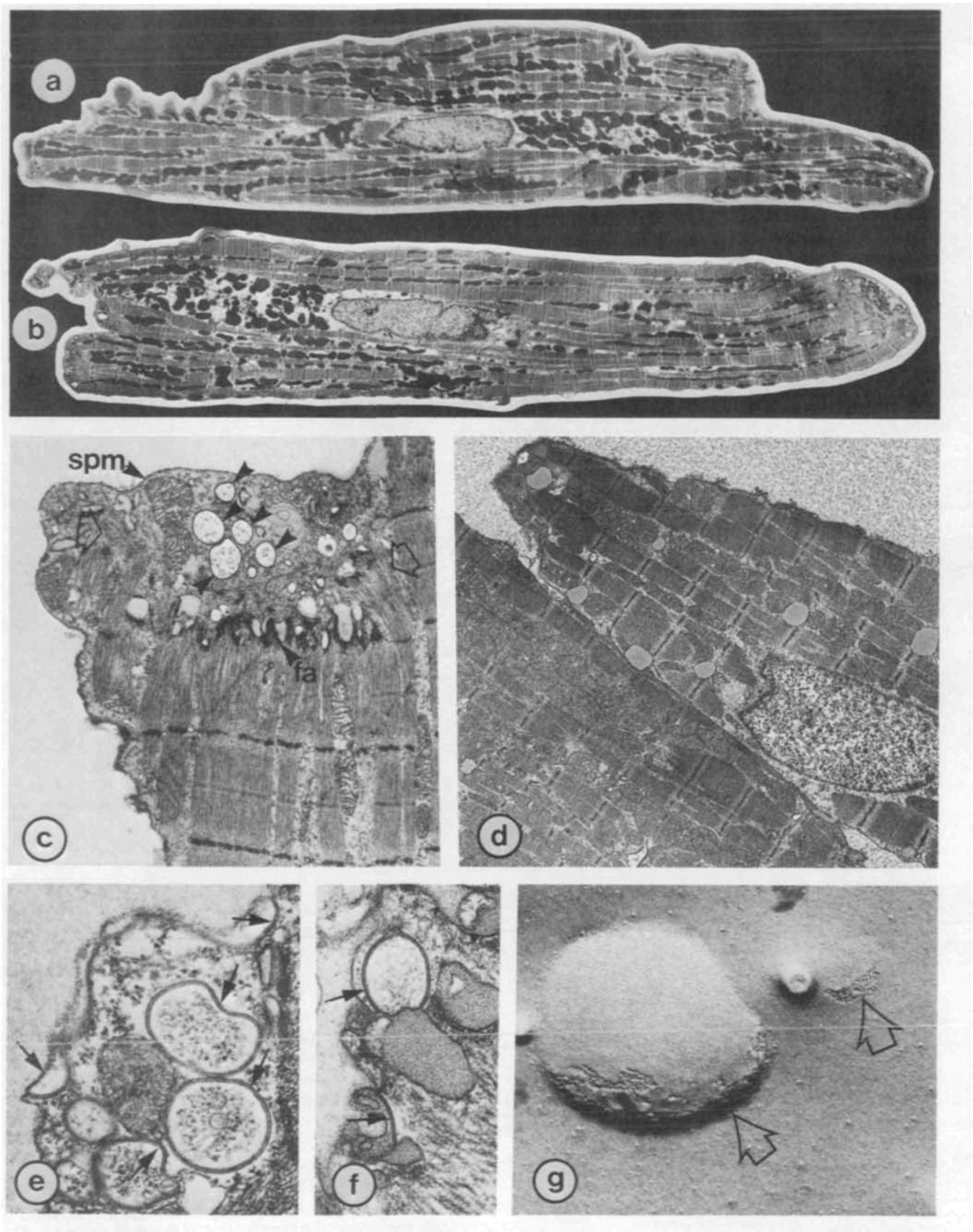


TABLE 1. Relative Numbers of Surface-Located Versus Annular Gap Junctions

Species	Time (hours)	Number of surface-located gap junctions (%)	Number of annular gap junctions (%)	Number of gap junctions analyzed
Rabbit	0	29	71	252
	22	23	77	237
Cat	0	54	46	46
	15	44	56	39
Guinea pig	0	23	77	123
	5	8.5	91.5	129

Statistical comparison by χ^2 test on original data, converted here to percentage values for ease of comparison.

Table 2 shows that a slight reduction in surface-located junctions and a slight rise in annular profiles occurred after 30 minutes' hypoxia, but this difference was not statistically significant ($p>0.05$). Analysis of gap junction distance from the cell surface in four matched pairs of samples (each from a different heart) also showed no significant difference between the hypoxic and control cells (Figure 10). These findings suggest that hypoxia does not stimulate internalization of gap junction membrane in isolated myocytes, at least under the conditions of the present experiments.

Is Gap Junction Membrane Degraded?

The possibility that internalized gap junction membrane is degraded was examined by measurement, over time after dissociation, of the relative quantity

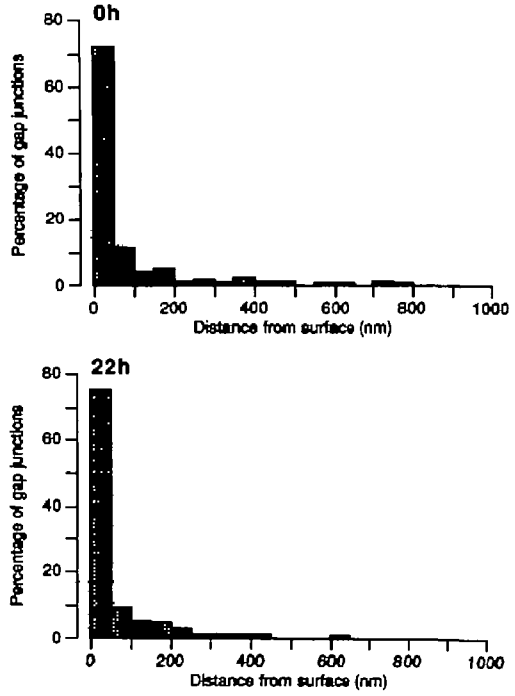


FIGURE 5. *Frequency distributions of measured gap junction distances from cell surface in isolated rabbit myocytes maintained for 0 and 22 hours.*

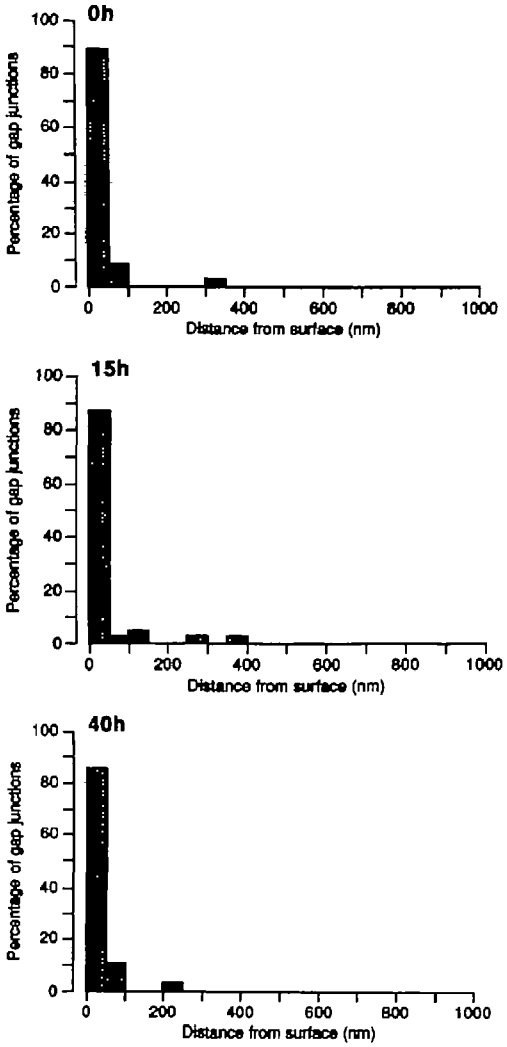


FIGURE 6. *Frequency distributions of measured gap junction distances from cell surface in isolated cat myocytes maintained for 0, 15, and 40 hours.*

of gap junction membrane and the incidence of "complex structures" (i.e., annular profiles showing complex morphological features, previously suggested to represent degradative change[22]).

Amount of gap junction membrane: Diameter of annular profiles. To estimate the relative quantities of gap junction membrane, we first measured the diameters of annular gap junction profiles (Table 3). No significant alteration occurred with time in any of the species investigated ($p>0.05$). However, the fact that the diameter of annular profiles appeared unchanged did not exclude the possibility of a decrease in the total number of gap junction structures. As an index of this, we counted the numbers of annular profiles and surface-located junctions visible per cell section, and again no significant difference ($p>0.05$) was found in any of the species over time. This comparison was carried out on the same cells used to obtain the data in Figures 5–7, that is, on cells preselected for the presence of at

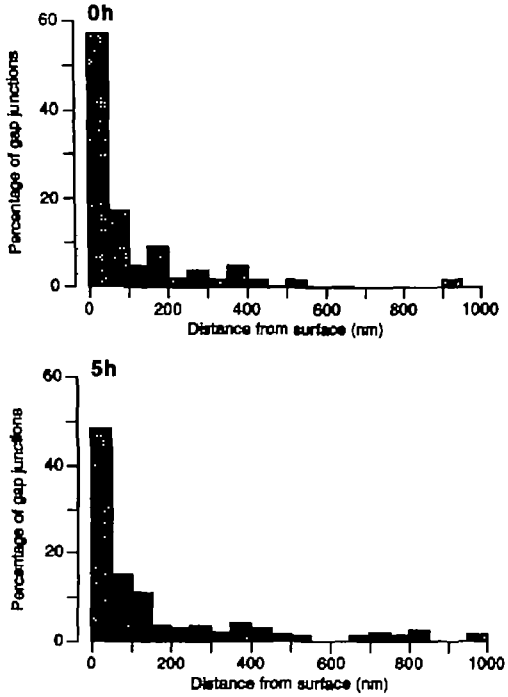


FIGURE 7. *Frequency distributions of measured gap junction distances from cell surface in isolated guinea pig myocytes maintained for 0 and 5 hours.*

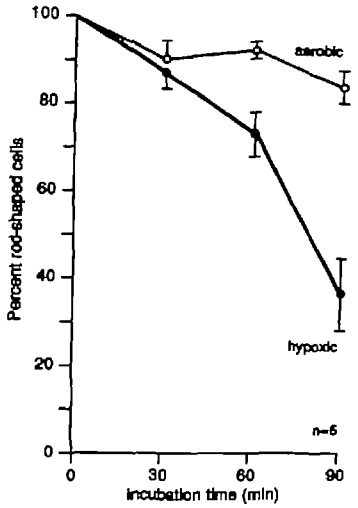


FIGURE 8. *Effect of hypoxia on isolated rat myocytes. The percentage of "rod-shaped" cells declines progressively under hypoxic incubation* (●)*, but falls only slightly in aerobic controls* (○) *over a 90-minute period. Percent of "rod-shaped" cells is normalized to 100% at 0 minutes.*

least one visible gap junction in the section plane. Therefore, it was necessary to check that, overall, the number of cell sections revealing one or more gap junctions versus the number of cell sections showing no visible junctions in the original random sections had remained unchanged. This was confirmed to be so (Table 3). Taken together, these findings suggest that the quantity of ultrastructurally detectable gap junction membrane did not change markedly over the periods studied in any of the species examined.

Amount of gap junction membrane: Gap junction profile length. Although the above findings, in contrast with previous reports,[10,22,23] point to the persistence of substantial amounts of gap junction membrane over the culture periods examined, the diameter of annular profiles and associated data provide only a crude guide to the relative quantities of gap junction present. As a separate analysis, therefore, we measured gap junction profile length in further samples of randomly sectioned cells whose sectional areas (as an index of relative cell volume) and plasma membrane profile lengths (as an index of relative surface area) were also determined. The data in Table 4 show that the sectional area and the plasma membrane profile length of these randomly sectioned cells did not alter significantly ($p>0.05$) between the freshly isolated and cultured samples; thus, the cells did not appear to change markedly in volume or in surface area over the time points studied. The mean profile length of gap junctions and the mean number of gap junctions observed per random section through a cell also remained remarkably constant. Calculation of the mean profile length of gap junction membrane per unit area of sectioned cell (a comparative index of gap junction surface area per unit cell volume) and of mean profile length of gap junction membrane per measured profile length of plasma membrane (a reflection of gap junction surface area in relation to cell surface area) also produced similar values in each case. It is conceivable that the various sources of inaccuracy inherent in measurements and calculations of this type may have concealed real changes in cell size or quantity of gap junction membrane. However, if such undetected changes do in fact occur, the results nevertheless indicate persistence of substantial quantities of gap junction membrane.

Incidence of "complex structures." "Complex structures" differ from the simple form of annular gap junction by containing additional membranous structures within or exterior to the vesicle lumen or between its limiting membranes (Figure 11). In the present study, the incidence of "complex structures" was low in all species and did not alter significantly ($p>0.05$) over the time periods examined (Table 5). This finding prompted us to reevaluate the significance of structural variation in annular gap junctions.

Ultrastructure of "complex structures." The typical simple spheroidal form of annular gap junction may either have an electron-lucent lumen or contain granular material (Figure 11a) identified as cytoplasmic matrix components, derived from the formerly adjacent myocyte, that became entrapped by the sealing-over process.

One form of "complex structure" consists of one or more profiles of gap junction membrane within an annular gap junction (Figures 11b, 11c, and 11e).

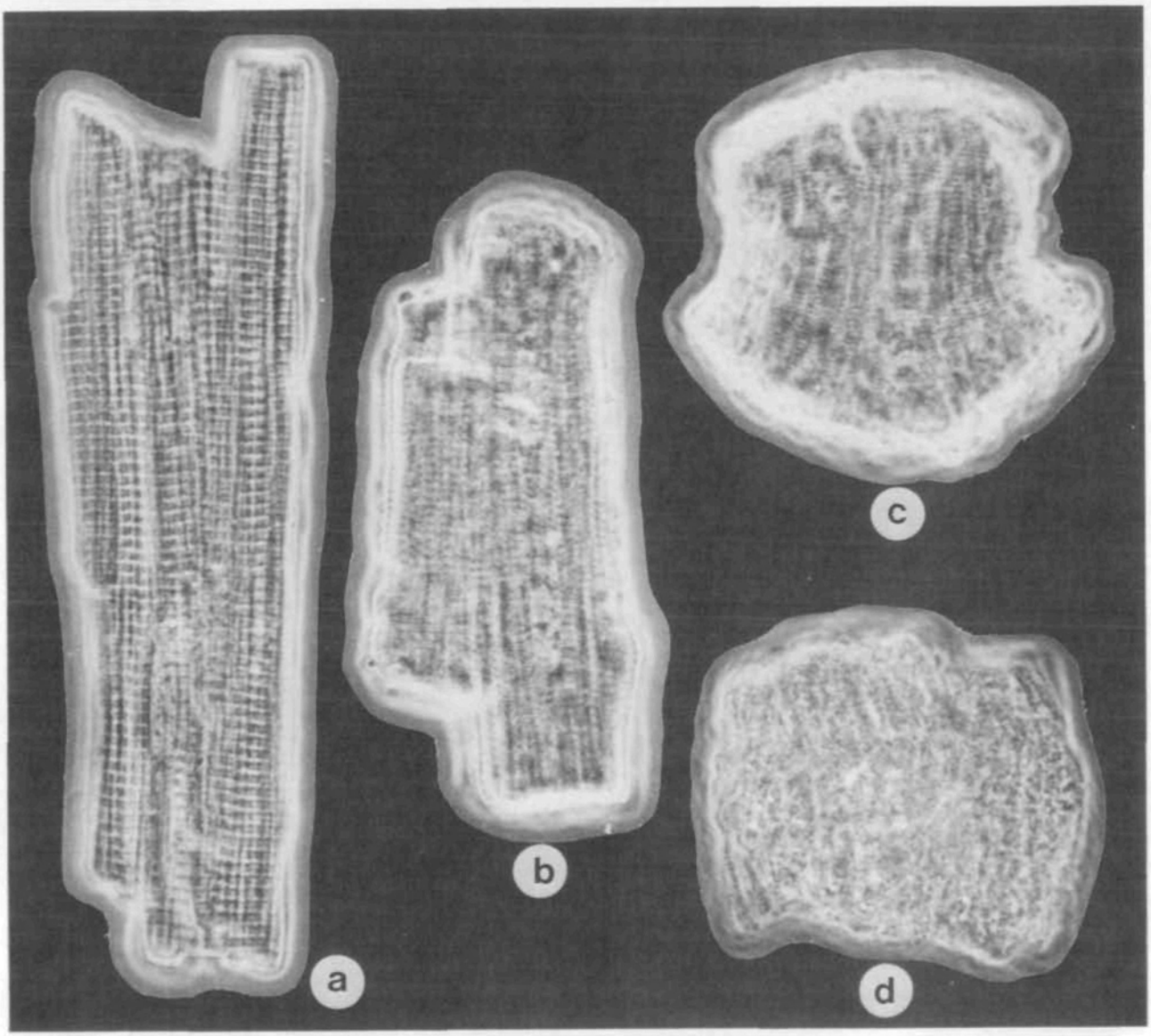


FIGURE 9. *Phase contrast light microscopy depicting response of isolated rat myocytes to hypoxic incubation.* Panel a: *Normal "rod-shaped" cell at 0 minutes.* Panel b: *Shortened "rod" form showing a reduction of approximately 33% in sarcomere length, typical of 30 minutes' hypoxic incubation. Contraction to this temporarily stable form is the first obvious sign that cells have responded to hypoxia.* Panels c *and* d: *Hypercontracted cells become progressively more numerous as period of hypoxic incubation is prolonged. Magnification* ×976.

Rather than representing portions of membrane detached from the gap junction vesicle's wall and shed into its lumen, favorable sections revealed that these images arose from complex involution of large surface-located or cytoplasmic gap junctions (Figure 11d). Thus, granular components within the inner of two concentric annular gap junction profiles are part of the cytoplasm in which the "complex structure" occurs.

The most common form of "complex structure" comprises an apparent gap junction vesicle containing one or more single membrane profiles in its lumen (Figure 11e–11j). Several plausible explanations, independent of the junction breakdown hypothesis, exist for these structures. First, sealing of the membrane margin around the torn-out junction segment may trap portions of intracellular membrane systems (e.g., sarcoplasmic reticulum) in addition to cytoplasmic matrix. Secondly, fragments of vesiculated plasma membrane generated during the tearing process may be similarly entrapped. Thirdly, one or both of the nonjunctional membrane portions of the annular gap junction wall (derived from the sealing membrane and the corresponding segment of plasma membrane from the cell that has endocytosed the junction) may infold (Figure 11k) in a similar manner to that shown for gap junctional membrane (Figures 11c and 11d), creating the impression of discrete membrane profiles in some section planes. It is also possible that these nonjunctional

TABLE 2. Effect of Hypoxia on Relative Numbers of Surface-Located Versus Annular Gap Junctions in Isolated Rat Myocytes

Treatment	Number of surface-located junctions (%)	Number of annular junctions (%)	Number of gap junctions analyzed
Control (30 minutes aerobic incubation)	22.5	77.5	71
Hypoxia (30 minutes)	17.3	82.7	81

membrane segments are inherently unstable and "bleb" into the gap junction vesicle interior. Finally, where nonjunctional membrane profiles are observed within the inner of two concentric annular gap junctions (Figure 11c), these are most likely to represent intracellular membrane components of the cell under examination. A similar set of explanations could account for the presence of membranous structures between the two (nonjunctional) membranes of the vesicle wall (Figure 11h).

Convincing instances of separation of the junctional membranes of annular profiles—a proposed early event in degradation[22]—were not observed in the present study, although a variable space between the nonjunctional membranes of the vesicle wall was not uncommon* (Figures 11g, 11h, and 11j–11l). The quantity of nonjunctional membrane in surface-located junctions can be substantial, exceeding that of the junction itself (Figure 11m); endocytosis in such cases produces gap junction vesicles composed predominantly of nonjunctional membrane. The portion of nonjunctional plasma membrane pinched off during endocytosis may not precisely match the adjacent sealing segment, and in the absence of any adhesive structure between these two membranes, a space of variable width between them is not unexpected.

Observations on the association of mitochondria with annular profiles of gap junction membrane provide further support for these interpretations. Gap junctions commonly occur adjacent to mitochondria in the intact heart, sometimes partially

*Experimental splitting of gap junctions (e.g., in 8 M urea, pH 10) shows that single gap junction membranes are readily distinguishable from nonjunctional membranes by their higher electron density.

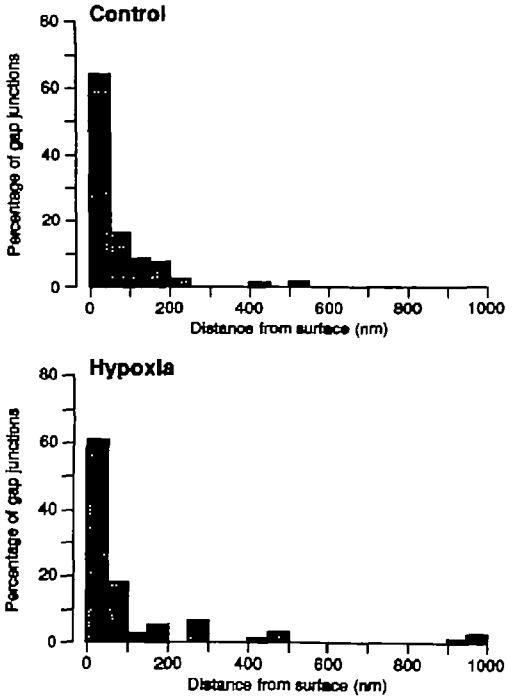


FIGURE 10. *Frequency distributions of measured gap junction distances from cell surface in isolated rat myocytes maintained for 30 minutes under aerobic (control) and hypoxic conditions.*

TABLE 3. Estimates of Quantity of Gap Junction Membrane

Species	Time (hours)	Diameter of apparent gap junction vesicles (nm)*	Number of gap junctions per cell section†	Number of cell sections containing gap junctions (%)‡
Rabbit	0	480±25×310±15 (n=100)	2.52 (n=100)	45.2 (n=250)
	22	520±29×351±19 (n=100)	2.37 (n=100)	44.4 (n=250)
Cat	0	509±66×382±50 (n=20)	2.3 (n=20)	36.0 (n=100)
	15	478±106×358±80 (n=20)	1.95 (n=20)	39.0 (n=100)
Guinea pig	0	426±28×311±19 (n=86)	3.07 (n=40)	48.5 (n=200)
	5	351±25×275±19 (n=86)	3.23 (n=40)	51.0 (n=200)

*Apparent gap junction vesicles are often ovoid; hence two diameter measurements were taken. First figure gives mean longest diameter, and second shows mean maximum diameter taken at right angles to first measurement. In each species, the diameters of apparent gap junction vesicles in myocytes maintained after isolation did not differ significantly from those of freshly isolated cells ($p>0.05$). Measurement of profile lengths of gap junction membrane in the same samples similarly showed no significant difference between time points. Statistical comparison by Student's t test; values are mean±SD; n=number of gap junctions measured.

†Data represent mean number of gap junctions (surface-located and annular profiles) present in cell sections preselected for presence of at least one gap junction. Statistical comparison by χ^2 test on original data; n=number of cell sections.

‡Data show percentage of cell sections revealing one or more gap junctions, expressed against total number of cells viewed in the sections. Only entire unobstructed (two-dimensional) views of cells were analyzed. Statistical comparison by χ^2 test on original data; n=number of cells analyzed.

TABLE 4. Comparison of Relative Cell Volume, Gap Junction Profile Length, and Relative Quantities of Gap Junction Membrane per Unit Cell Volume and per Unit Cell Surface Area

	0 Hours	22 Hours
Mean area of sectioned cell (μm^2±SEM)	288.5±18.9	286.1±21.8
Mean profile length of plasma membrane/sectioned cell (μm±SEM)	86.2± 3.9	81.6± 4.2
Mean profile length of gap junctions (μm±SEM)	1.67±0.94	1.42±0.76
Mean number of gap junctions per cell section	0.83	0.9
Mean profile length of gap junction per unit area sectioned cell (nm/μm^2)	4.8	4.6
Mean profile length of gap junction per unit profile length of plasma membrane (nm/μm)	16.11	16.24

Data are from randomly sectioned rabbit myocytes (n=200), derived from four different hearts and maintained for 0 and 22 hours, from a separate sampling exercise from that providing the data in Table 3.

TABLE 5. Incidence of Complex Structures

Species	Time (hours)	Number of "complex structures" (% of gap junction population)
Rabbit	0	2.4 (n=252)
	22	5.9 (n=237)
Cat	0	6.5 (n=46)
	15	10.3 (n=39)
Guinea pig	0	10.6 (n=123)
	5	7.0 (n=129)

Statistical comparison was by χ^2 test on absolute numbers of "complex structures" and absolute numbers of gap junctions not classified as "complex"; data are expressed as percentage values in the table for ease of comparison. n=number of gap junctions analyzed.

enveloping them to give annular profiles in appropriate section planes.[35] The presence of mitochondria within the simple form of annular gap junction in isolated myocytes (Figures 12a and 12b) would seem to confirm that the junction sealing process can indeed entrap large portions of the neighboring cell's cytoplasm. Once within a cytoplasmic gap junction vesicle, mitochondrial membranes are prone to "blebbing" (Figure 12b); hence, it is not surprising that less easily identified membranes from other sources appear disrupted or abnormal (Figure 11f). Mitochondria within the inner of two concentric annular gap junction profiles can be demonstrated to belong to the cell under examination (Figures 12c and 12d), and further infolding of the junctional membranes can give rise to yet more complex images (Figure 12e).

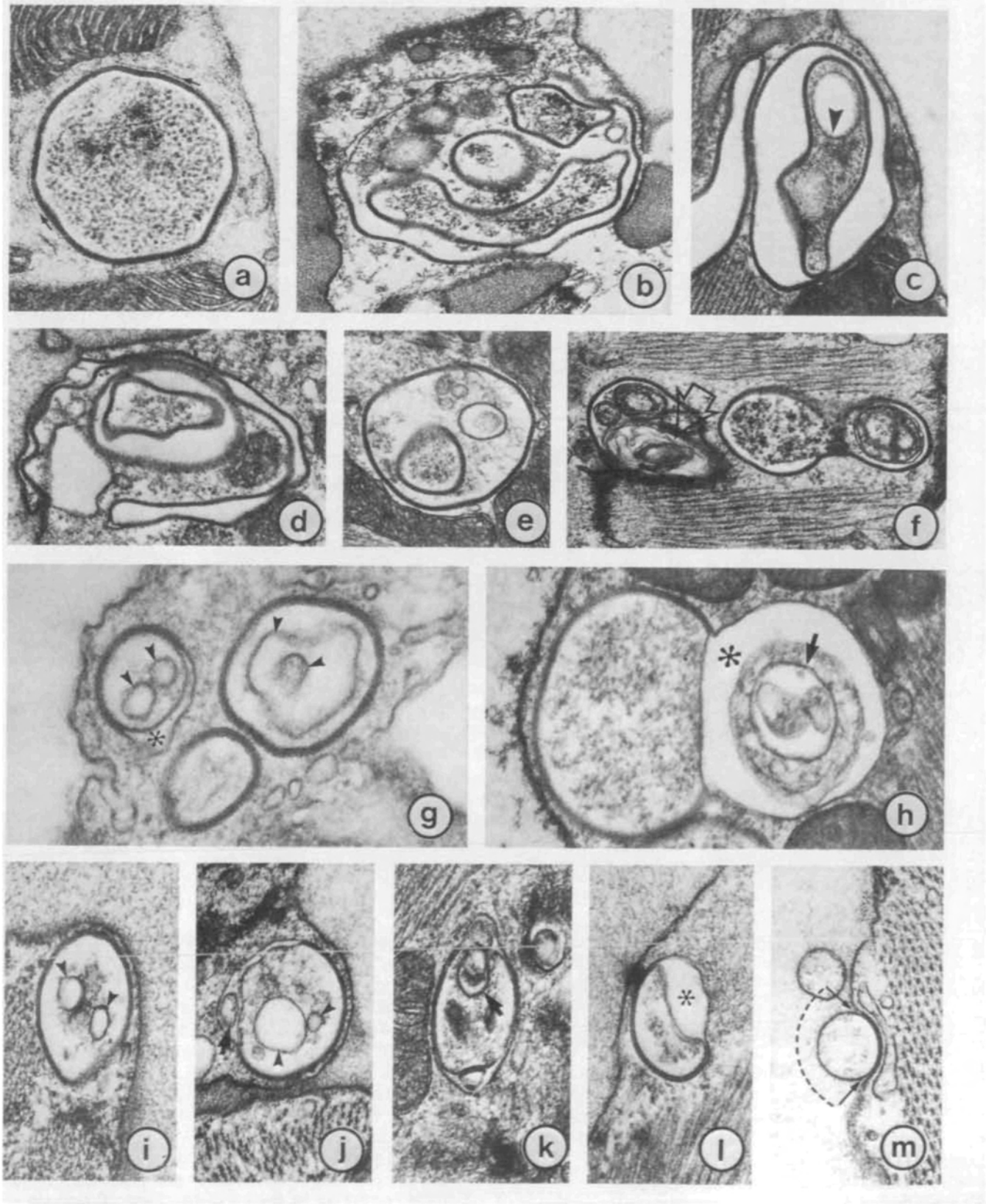
Annular profiles of gap junction membrane associated with possible lysosomal structures were very rare. Figure 12f shows one example, which could be interpreted as a cytoplasmic gap junction vesicle lying within an autophagic vacuole, with two primary lysosomes nearby. It should be emphasized that such images were most exceptional, accounting for fewer than 0.001% of annular gap junction profiles examined.

Discussion

The findings of the present study extend earlier observations[15–22] and provide a more detailed picture of the fate of gap junctions in dissociated and cultured adult heart muscle cells. Although the longitudinal splitting of gap junctions into their component single membranes has been demonstrated after hypertonic perfusion of liver[36] and discussed as one possible mechanism in separation of myocyte intercalated disks,[17–19] the evidence overall is that this mode of separation seldom occurs with conventional dissociation procedures, either in the heart[15,16,20–22] or other multicellular systems.[37,38] Instead, when two cells are dissociated, intact bimembranous gap junctions are retained

FIGURE 11. *Morphology of "complex structures."* Panel a: *Simple form of annular gap junction containing diffuse granular material. Note faint, discontinuous electron-dense line around outer surface (arrows), possibly corresponding to filamentous "basket" structure.*[34] Panels b *and* c: *Examples of apparent gap junction vesicles containing gap junction membrane. Single membrane structure (arrowhead) in panel c is situated in cytoplasm of cell under examination.* Panel d: *Large surface-located junctions, as in this example, tend to collapse upon themselves, forming a series of complex infoldings rather than an indentation of regular curvature. Cross-sections of this structure would result in images similar to those of panels b and c.* Panel e: *Apparent gap junction vesicle containing both gap junctional and nonjunctional membrane.* Panel f: *Occasionally, complex structures may be dumbbell shaped or they may contain lamellar structures (open arrow), presumably derived from membranes trapped in junctional vesicle interior.* Panels g *through* j: *Single membrane structures may occur in annular junction lumen (arrowheads) or between nonjunctional membrane portions of vesicle wall (arrows).* Panel k: *Involution (arrow) of nonjunctional membrane portion of wall of annular junction profile.* Panel l: *Wide space (*) between nonjunctional membrane segments of wall of a simple annular junction. (Similar separations (*) between nonjunctional membrane wall segments are also noted in panels g and h.)* Panel m: *Simple form of surface-located junction illustrating quantity of nonjunctional membrane in sealed loop (between arrows). Upon internalization this nonjunctional membrane and a corresponding portion of endocytosed plasma membrane would form the greater part of annular gap junction wall. Magnification: a, ×52,200; b, ×46,000; c, ×41,400; d, ×41,400; e, ×43,200; f, ×41,400; g, ×86,400; h, ×61,600; i, ×50,400; j through m, ×52,200.*

by one cell at the expense of its neighbor. Some gap junction plaques appear to be torn out at, or very close to, their border and internalized rapidly to form vesicles consisting (as far as can be judged by serial sections) almost entirely of junctional membrane. In many gap junctions, however, tearing occurs at some distance from the junctional plaque, creating a margin of nonjunctional membrane that



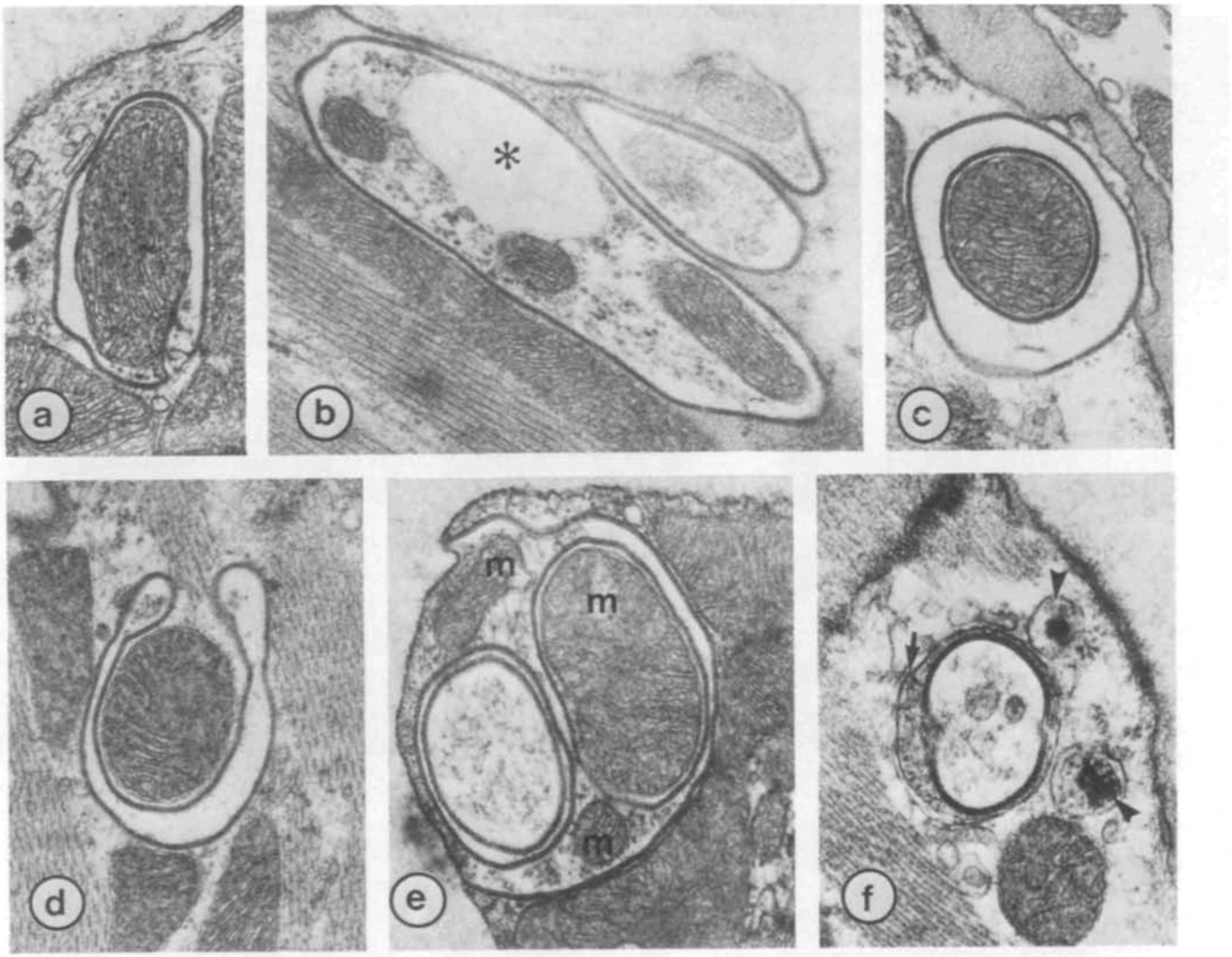


FIGURE 12. Panel a: *Simple form of apparent gap junction vesicle containing a single mitochondrion.* Panel b: *Large gap junction structure containing three mitochondria, one of which shows a large bleb of its outer membrane* (*). Panel c: *Mitochondrion within inner of two concentric annular gap junction profiles.* Panel d: *Annular gap junction structure wrapped around a mitochondrion, partially enclosing it. Cross-section of this structure would result in an image similar to that in panel c.* Panel e: *More complex gap junction–mitochondria associations. A surface-located gap junction contains two small mitochondria (m) and two annular gap junction profiles. One of these annular profiles contains a mitochondrion and the other yet another annular gap junction.* Panel f: *Rare example of an annular gap junction profile enclosed by a membrane (arrow), possibly representing an autophagic vacuole. Two smaller membrane-bound bodies (arrowheads) containing electron-dense material may represent primary lysosomes. Magnification: a, ×46,800; b through d, ×43,200; e, ×39,600; f, ×52,200.*

fuses with itself to form a continuous sheet, effectively sealing off the otherwise exposed junctional surface. The damage inflicted by the tearing process might seem inconsistent with the high yields of calcium-tolerant, functionally intact myocytes routinely obtained,[27-31] especially in view of the abundance of gap junctions between myocytes.[5,39] What appears to happen is that "junction-corresponding holes," like the exposed junctions themselves, may in some instances become sealed over by refusion of the torn plasma membrane.[21]

Annular gap junctions, which may represent cross-sectioned invaginations or cytoplasmic vesicles of gap junction membrane, have previously been reported in a wide range of cell types[40-58] including myocytes of intact myocardium.[59-62] One functional role suggested for cytoplasmic gap junction vesicles is as a mechanism for the removal and/or disposal of unwanted gap junction membrane, for example, during development, differentiation, and tissue remodeling.[50-58] Although it is important to bear in mind the distinction between such instances of gap junction endocytosis (which appears to be a normal physiological process of intact multicellular tissue systems) as opposed to gap junction endocytosis occurring in response to enforced cell disso-

ciation, some interpretations of the latter[17,22] have drawn parallels with the former. In one such study on the isolated adult myocyte, a sequence of events akin to that documented in tissue systems was proposed, involving progressive gap junction endocytosis and inward migration of gap junction vesicles, followed by their degradation and transformation into "complex structures."[22]

Our demonstration by serial sectioning that some (but not all[21]) annular gap junction profiles in freshly isolated myocytes are truly discrete vesicles accords with earlier lanthanum staining results,[22] and, when taken with the pronounced difference in the incidences of annular profiles between intact myocardium and freshly dissociated cells, confirms that a major proportion of the gap junction population is internalized at the time, or very shortly after, the cells become separated. The variation in degree of invagination shown by the remaining surface-located junctions could be interpreted as evidence that such an internalization process actively continues during and after cell isolation. Our analysis of the ratio of surface-located-to-annular junctions between time points in culture suggests that further net endocytosis may well have taken place in the guinea pig myocytes, but there is no evidence that it did so in the rabbit and cat cells, even when they were maintained for much longer periods under the same conditions. Whether species differences in the response to dissociation conditions have any bearing on this apparent difference in gap junction behavior requires further study.

The incidence of annular profiles of gap junction membrane is reportedly increased by hypoxia in some nonmyocyte cell types in intact tissue.[63,64] Bearing in mind the fundamental differences between a natural multicellular system and artificially dissociated cells, our finding that hypoxia did not appear to augment further the marked gap junction internalization already induced by dissociation in the myocyte is, perhaps, not surprising.

Substantial inward migration of gap junction vesicles, reported to be a sequel to gap junction internalization in isolated rat myocytes maintained in culture medium,[22] did not occur in our rabbit and cat cells. Although, in concert with the continuing endocytosis, some inward movement was detected in guinea pig myocytes, it should be stressed that the distances involved were small, corresponding on average to less than the thickness of the twinned membranes of four side-by-side gap junctions.

A demonstrable decrease in the quantity of gap junction membrane, or its complete disappearance, as reported after culture of attached myocytes for 12 or 24 hours,[10,23] would provide clear evidence for junction degradation. However, the interpretation of our finding that there appeared to be no diminution in the number or size of gap junctions or in the total quantity of gap junction membrane in our cells is not so straightforward. New gap junction formation between dissociated myocytes brought back into contact has been reported to occur after overnight incubation[13] or even earlier (4 hours[26]); therefore, it might be supposed that a balance between breakdown and synthesis could maintain a constant quantity of gap junction membrane in our cells. However, we found only occasional new side-by-side contacts in our 22-hour cells, and if new gap junctions were present in these zones, they were too small to make any contribution to the thin section measurements. Thus, our data appear consistent with an absence of gap junction breakdown. However, as it has been shown that experimental proteolytic digestion of isolated liver gap junctions can lead to considerable degradation of the connexon subunit polypeptide without alteration to the junction's thin section profile,[65] our ultrastructural results cannot rule out breakdown of this nature.

To examine the degradation concept further, we investigated the occurrence and nature of "complex structures"—the category of myocyte annular profile whose structural features have been considered indicative of breakdown.[22] Although "complex structures" are reported to become common after overnight incubation of myocytes,[22] we found that their initial relatively low incidence in freshly isolated cells did not increase significantly over the time periods studied. Moreover, careful inspection of the ultrastructure of the "complex structures" revealed that virtually all could be explained independently of the degradation hypothesis if account was taken of such factors as 1) the trapping, in the vesicle interior, of cytoplasmic and membrane inclusions originating from the neighboring cell, 2) the presence of nonjunctional membrane in addition to junctional membrane in the vesicle wall, and 3) the extensive folding that larger junctions may undergo. We found that in isolated myocytes, the sequestration of an annular junction within a possible autophagic vacuole, as previously recorded in some tissues,[54,58,66] is an extremely rare event. The presence of gap junction membrane fragments within phagolysosomes and multivesicular bodies, as previously reported in various (nonmyocyte) cells of intact tissue,[51,52,58] would have strengthened the case for general lysosomal degradation, but none of the "complex structures" of the myocyte conformed to these distinctive forms of secondary lysosome.

Taken together, our findings do not entirely fit the concept that unneeded gap junction membrane in dissociated myocytes is necessarily rapidly removed by endocytosis for rapid degradation via a vesicle migration–lysosomal pathway.[22] The morphological changes that occur in mature myocytes after isolation vary quite remarkably in their time of onset, rate, and nature according to the culture method,[10,24] and this may lie behind some of the differences between our results and those of others.[22,23,26] Furthermore, although upon dissociation of some nonmyocyte cell types, gap junction tearing and sealing follow the same pattern as that of myocytes,[37,38]

there is no compelling evidence that endocytosis of the surface-located junctions in these systems must inevitably follow. For example, hepatocytes rapidly lose their surface-located gap junctions after dissociation[67] and show annular profiles in culture,[68] but although annular profiles have also been demonstrated in Novikoff hepatoma cells,[50] the endocytosis of surface-located junctions in these cells after dissociation is reported to be rare.[38]

If, as appears, gap junctions are sealed over, there is little reason to suppose that survival of an isolated cell is threatened by the presence of surface-located junctions; therefore, the cell may have no urgent need to rid itself of them, disposal proceeding no faster than the normal turnover rate. The half-life of the liver gap junction protein is exceptionally short (3–5 hours)[69] compared with other membrane proteins, but the heart gap junction protein, though showing some homology, differs from that of liver (43 kDa and 32 kDa, respectively[5–7,70–72]), and there is no evidence to date that its turnover is as rapid. It is noteworthy that even in some tissue systems, endocytosed gap junctions appear to persist for prolonged periods.[54]

That tissue systems have the capacity not only to internalize but also to degrade cytoplasmic gap junction vesicles as a mechanism for the reduction and disposal of gap junctions is now well documented in a wide variety of cell types,[50–58,66] but extrapolation of such a scenario to the gap junction structures viewed in artificially dissociated cells does not appear to be justified, at least with the evidence so far available. Another possible mechanism discussed for removal of gap junctions in tissues is dispersal of connexons in the membrane plane,[63,73–75] a reversible process that facilitates rapid reassembly.[76] Whether dispersed connexons (or their polypeptide subunits) are withdrawn (or inserted) directly or by vesicle fission (or fusion) events, and whether degradation can occur in situ in the plasma membrane, are not known. Whatever the details of this process, there is some evidence that connexon dispersal is the preferred mechanism of disassembly where cell dissociation splits the junction into its two component single membranes.[75,77] If this view is correct, then it is not surprising that the bimembranous gap junctions in our dissociated myocytes show no signs of this process.

Our results do not exclude ultimate degradation and disappearance of gap junction membrane in isolated myocytes maintained for culture periods longer than those examined here. If such breakdown does eventually occur, then the time scale involved is clearly substantially longer than that previously envisaged for the myocyte. This remarkable persistence of gap junction membrane in the mature differentiated myocyte leaves open the possibility that the cardiac muscle cell gap junction protein has a longer half-life than its counterpart in liver.

Acknowledgment

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