THE FINE STRUCTURE AND ELECTROPHYSIOLOGY OF HEART MUSCLE CELL INJURY

KATE M. BALDWIN

From the Department of Biological Structure, University of Washington, Seattle, Washington 98105

ABSTRACT

Injured frog heart cells electrically uncouple from their uninjured neighbors within 30 min after injury. This uncoupling process can be shown by the disappearance of an injury potential measured between such injured and uninjured cells. In the present study, the time course of the decline of injury potentials, and thus of electrical uncoupling, in bullfrog atrial trabeculae was determined. Tissue was fixed with glutaraldehyde and osmium tetroxide at various times after injury to determine the morphological changes which accompany this uncoupling process. In some cases, ruthenium red was included in the fixatives. Normal atrial cells are long and narrow, with intercellular junctions located along the lateral surfaces of the cells. Two types of intercellular junctions have been observed: cardiac adhesion plaques (CAPs), and close junctions. Close junctions occur only infrequently. Ruthenium red penetrates all around the cells, leaving only small areas within the CAPs unstained. After injury, the cells are very dense and the myofilaments disarranged. Both types of intercellular junction remain intact, and only slight changes within CAPs are observed. The results are discussed in relation to current concepts of intercellular communication.

INTRODUCTION

The exact mechanism of intercellular impulse-conduction in cardiac muscle is still in question. It is known that conduction of excitation proceeds electrotonically through low electrical-resistance pathways between cardiac cells (Barr and Berger, 1964; Barr et al., 1965; Tillie, 1966; Van der Koot and Dane, 1964; Weidmann, 1952, 1965, 1966; Woodbury, 1962; Woodbury and Grill, 1961; Woodbury and Gordon, 1965), but the nature and location of such pathways is uncertain. Barr et al. (1965) suggest that tight junctions are the sites of low electrical resistance in cardiac muscle. Loewenstein (1966, 1968), Loewenstein et al. (1967), and Loewenstein and Penn (1967) propose that specific patterns of Ca++ distribution at tight and septate junctions are responsible for the low electrical-resistance between epithelial cells. In the present study, the problem of electrical communication between cardiac cells was investigated by comparing the morphology of electrically coupled cells to that of electrically uncoupled cells.

Injured frog heart cells become electrically isolated from their uninjured neighbors usually within 15-30 min after the injury is inflicted (Baldwin et al., 1963; Henry et al., 1961). This uncoupling process can be measured by the decrease of an injury potential, because injury potentials depend upon the existence of low-resistance connections between the injured and uninjured cardiac cells, and their decline with time is due to the gradual loss of such connections.
The experimental plan was to injure one-half of a bullfrog atrial trabecula, measure the resulting injury potential, fix the trabecula at some time during the decline of the injury potential, and then examine it by electron microscopy. Since no detectable morphological change occurred in synchrony with the injury-potential decline, cells were examined only in the coupled state (when the injury potential is at its maximum), and in the uncoupled state (when the injury potential has decreased to zero).

Bullfrog atrium is a convenient tissue to use for this study because the wall is composed largely of trabeculae which run more or less freely for several millimeters. It is therefore possible to remove a strip of myocardium (for electrical recordings) with damage only at the ends, and to insure that nearly all the injury sustained by the tissue is that which is purposely inflicted.

MATERIALS AND METHODS

Dissection Procedure

Adult bullfrogs (Rana catesbeiana) were chemically pithed by injecting 0.1 ml of 2% xylocaine hydrochloride into the brain case. The isolated heart was placed in Ringer's solution, and the dissected trabecula was placed on one side of the diaphragm. The free end of the silk thread, which had been tied to the trabecula, was threaded through a needle which was used to pierce the diaphragm and to pull about half the trabecula through the hole. This half was used as the injured part because a small amount of injury may have occurred in these manipulations. The major injury was crush, produced in each case by squeezing the tissue several times with a pair of forceps. The difference in potential between the solutions bathing the injured and uninjured muscle was measured with the aid of Ringer's-agar salt bridges, Hg-HgCl half-cells in saturated KCl and an amplifier and recorder (Fig. 1). The base line for injury-potential measurements was obtained by placing the two salt bridges in the same side of the trough.

Electrophysiology

The experimental arrangement for measuring injury potentials is shown schematically in Fig. 1. The recording wire was a polyethylene trough which had been cut in half and glued back together with rubber cement, so that a thin rubber diaphragm stretched between the two halves. The two halves of the chamber were filled with amphibian Ringer's solution, and the dissected trabecula was placed on one side of the diaphragm. The free end of the silk thread, which had been tied to the trabecula, was threaded through a needle which was used to pierce the diaphragm and to pull about half the trabecula through the hole. This half was used as the injured part because a small amount of injury may have occurred in these manipulations. The major injury was crushing, produced in each case by squeezing the tissue several times with a pair of forceps. The difference in potential between the solutions bathing the injured and uninjured muscle was measured with the aid of Ringer's-agar salt bridges, Hg-HgCl half-cells in saturated KCl and an amplifier and recorder (Fig. 1). The base line for injury-potential measurements was obtained by placing the two salt bridges in the same side of the trough.

Electron Microscopy

The fixative was applied to the tissue at one of three different stages: (a) the fixative was injected into the sinus opening of the still beating heart before the heart wall was cut open, the atria were then opened and the tissue flooded with additional fixative; (b) the opened atria were flooded with fixative at various times after some trabeculae had been injured in situ, but before any trabeculae had been cut away from the heart wall; or (c) the fixative was added to the polyethylene chamber after recording injury potentials. In all cases, the initial fixative used was 0.88% glutaraldehyde in 0.067 M cacodylate buffer at pH 7.2, which is isotonic for amphibians.

A standard procedure was then followed: the isolated trabeculae were fixed for 1 hr in the cacodylate-buffered glutaraldehyde at 0°C, rinsed with 0.125 M cacodylate buffer for 15 min, and then postfixed in cacodylate-buffered OsO4 for 1 hr at 0°C. The tissue was dehydrated in ethanol and embedded in Epon (Luft, 1961). Unless otherwise specified, sections were double-stained with saturated uranyl acetate and lead citrate (Reynolds, 1963).

Two variations of the above procedure were also
used. Some tissue was stained en bloc with 0.5% uranyl acetate in collidine buffer at pH 6.1 (Trelstad et al., 1966). Other tissues were fixed in solutions containing ruthenium red (Luft, 1965). For these experiments, the tissue was fixed for 1 hr in a solution containing equal volumes of 2.62% glutaraldehyde, 0.188 M cacodylate buffer, and ruthenium-red stock solution (1500 ppm in distilled water) at 0°C. It was then rinsed for 15 min in 0.125 M cacodylate buffer and postfixed in a solution containing equal volumes of 5% OsO4, 0.188 M cacodylate buffer, and ruthenium-red stock solution (1500 ppm in distilled water) for 3 hr at room temperature. After a brief rinse in the buffer, the tissue was then dehydrated as in the standard procedure (Luft, 1965).

RESULTS

Electrophysiology

Injury potentials recorded from single atrial trabeculae showed a time course of decay similar to that reported for frog ventricle (Baldwin et al., 1963; Engelmann, 1877; Henry et al., 1961; Rothschuh, 1951). Initial injury-potentials of 4–19 mv usually decayed completely within $\frac{1}{2}$ hr (Fig. 2). Once the injury potential had reached a low value, reinjury of the previously injured area had no effect on the injury potential. However, the injury potential could be renewed to near its former value if a new injury was made close to the old injury. The injury potential was positive when the injured side was grounded.

Electron Microscopy

DEFINITION OF TERMS: Trabeculae fixed initially by injecting buffered glutaraldehyde into the sinus opening of the heart are referred to as normal tissue. Tissue fixed in this way provided a basis on which to judge control tissue (see below) as to quality of fixation, damage due to mechanical manipulation, and the effect of leaving the tissue in Ringer’s solutions for the various times required by the experimental procedures.

Injured tissue was injured either in situ or in the recording chamber, and was fixed at different times after the injury was inflicted. A control sample was obtained for every injured sample.

Control tissue was taken either from an adjacent uninjured trabecula (when the injury was produced in situ) or from the uninjured end of an injured trabecula (when the injury was produced either in situ or in the recording chamber).

NORMAL AND CONTROL TISSUE: As control tissue did not differ significantly from normal tissue, these two will be described together. The endocardial surface of the atrial wall was composed of many trabeculae which ran in different directions at different levels, forming a contractile meshwork. Trabeculae were composed of many small bundles of several muscle cells whose long axes were oriented parallel to the long axis of the trabecula. The bundles were each surrounded by a basal lamina (Fig. 3), and they formed branching and anastomosing strands within the trabecula. The individual cells did not branch. The fusiform cells were usually 3–12 $\mu$ in diameter and contained 1–4 myofibrils in cross-section. They were too long to be measured easily by electron microscopy, and their borders could not be seen by light microscopy. Barr et al. (1965) isolated atrial muscle cells from Rana pipiens by using ethylenediaminetetraacetate (EDTA) and found that they were 175–250 $\mu$ long.

Intercellular junctions oriented perpendicular to the myofibrillar direction, which are typical of the mammalian intercalated disc, were rare in frog atrial muscle. Most intercellular junctions were located along the longitudinal surfaces of the cells, often in association with Z bands. Such junctions, whether associated with myofibrils or not, were all similar morphologically and will be referred to as cardiac adhesion plaques, or CAPs (Figs. 3–5). Occasionally CAPs inter-
FIGURE 3 Normal tissue. Cross-section of a trabecula near its surface, illustrating the cells grouped together in bundles, each of which is surrounded by a basal lamina (BL). Endothelial cells (E), collagen fibers (co), nerve fibers (N), and cardiac adhesion plaques (CAP) are also shown. Unless otherwise indicated, the scale markers represent 1 μ × 7500.
**Figure 4** Control tissue, fixed 1 hr after injury to an adjacent trabecula. This longitudinal section illustrates the typical appearance of CAPs. Note the close association of the CAPs with Z bands. × 40,200.

**Figure 5** Control tissue, fixed 1 hr after injury to an adjacent trabecula. In this case a CAP is shown which is not associated with any myofibrils. Note the dense bars which can be seen within this CAP (small arrows). The intercellular dense line can be seen at large arrow. A close junction (cj) and a diadic coupling between the sarcoplasmic reticulum and the plasma membrane (D) are present. × 43,300.
ruptured myofibrils in a tangential or oblique manner. The area of cell surface that was involved in CAPs varied, but it appeared that CAPs preferentially located in rows along the length of the cell, rather than randomly around the cell (see Fig. 11).

The cell membrane appeared as a single dense line in all material fixed by the standard technique. When measured from the center of one cell membrane to the center of the adjacent cell membrane, the intercellular gap at a CAP was \( 270 \pm 25 \) A. With uranyl-acetate block staining, the trilaminar structure of the plasma membrane could be seen (Fig. 9). In uranyl-acetate block-stained material the spacing between the centers of the inner (cytoplasmic) leaflets of the two unit membranes at the CAP was \( 300 \pm 30 \) A, but between the centers of the outer leaflets of the two unit membranes the spacing was \( 190 \pm 30 \) A. This suggests that only the inner leaflets of the unit membranes were seen after using the standard technique.

CAPs were characterized by an accumulation of dense material both intracellularly and extracellularly. The extracellular density was finely granular in nature and showed no organization in sections perpendicular to the cell membrane, except for an occasional faint suggestion of a dense line in the center of the extracellular space (Fig. 5). Since this dense line could be seen in both longitudinal and cross-sectioned material, it probably represents a plate-like structure. The intracellular density associated with the CAP was usually finely granular also. However, a certain amount of organization, which was enhanced by uranyl-acetate block staining, could sometimes be detected (Figs. 5, 9). It appeared as dense bars (about 300 A long), which radiated into the cell perpendicular to the cell membrane. The reason for the variable, finely granular or structured, appearance of the CAP with the standard fixation is unknown. The dense materials comprising CAPs (intracellular portion) and Z bands were morphologically similar and often contiguous (Figs. 4, 11).

In tangential sections the CAP had a mottled appearance (Fig. 15). As illustrated in Fig. 16, the central part of this mottled region was extracellular, but at its periphery there appeared to be an intracellular portion as well. The intracellular mottling may be due to the dense bars mentioned above, cut in cross-section.

CAPs which have been pulled apart are rarely seen.

In addition to CAPs, another type of intercellular junction was infrequently observed. These close junctions had a slight increase in density of the adjacent cytoplasm and close apposition of the two cell membranes (Fig. 5, cj). The plasma membranes of the adjacent cells came very close together, but a 10–20 A gap was present between them (Fig. 9). Close junctions were rare in normal and control tissue, and when present were very short in extent (less than 0.1 \( \mu \)). However, in four (out of a total of 51) blocks of tissue, close junctions were exceptional in two respects: they were seen relatively frequently (though much less often than CAPs), and were longer than usual (up to 0.4 \( \mu \)). In addition, all four cases were characterized by swollen mitochondria and/or widely dilated sacs of endoplasmic reticulum (Fig. 19).

In three of the four cases, the other blocks of tissue which had been processed together with the exceptional tissues (in the same fixation bottle) did not have this aberrant appearance; in the other case, only one portion of the tissue block was abnormal.

**Electrically uncoupled injured tissue:** Injured tissue which was fixed \( \frac{1}{2} \) hr after injury appeared morphologically similar to that fixed at later times. This was true regardless of whether the injury and fixation were carried out in situ or whether the injury-potential decline was measured and the trabecula fixed in the recording chamber. Since the injured tissues from the in situ cases would be expected to be physiologically uncoupled, as were those in the trabeculae where injury potentials were actually recorded, all the tissues which were fixed \( \frac{1}{2} \) hr or more after injury will be described together.

The injured cells increased in density and their myofilaments were in disarray (Fig. 6). Often their outline was irregular and the intercellular boundaries formed tortuous paths. The normal banding pattern was either indistinct or lost altogether. The normal spacing between the thick filaments of 525 A as seen in cross-section decreased to 400 A in the injured cells. Mitochondria enlarged and clumped together.

Two changes with the CAP were observed in some, but not all, cases. A dense plaque appeared parallel to and approximately 60 A away from...
FIGURE 6 Electrically uncoupled injured tissue, fixed 50 min after injury (see curve 1, Fig. 2). The cell in the center of the field is typical of cells in the injured part of the trabecula. The particular field shown was taken from the boundary region between the injured and uninjured tissue. Typical of such boundary regions, cells with marked morphological alterations, such as the cell in the center of the field, were found adjacent to cells with little or no morphological alterations, such as the cell at the bottom of the field. A CAP and close junction (cj) can be seen. × 37,100.

The inside of the cell membrane (Fig. 7), and the dense line in the center of the extracellular space became more apparent (Fig. 7). There appeared to be no difference in the structure of the CAP whether it was located between two injured cells or between an injured cell and an uninjured cell, except that the course of the cell surface, and thus of the CAP, was often more tortuous between two injured cells.

There were no significant differences in the intercellular gap width at the CAP between the injured material and control tissue. After the standard technique, the intercellular gap (measured from the center of one cell membrane to the center of the adjacent cell membrane) was 270 ± 35 Å (control, 270 ± 25 Å). After uranyl-acetate block staining, it was 310 ± 40 Å and 190 ± 10 Å when measured between the inner (cytoplasmic) and outer leaflets of the unit membrane, respectively (control, 300 ± 30 Å and 190 ± 30 Å). The percentage of total cell-surface area involved in CAPs was not known for either control or...
injured tissue and therefore a quantitative comparison could not be made. However, no obvious change in area was noted, and only a very few CAPs were observed which had been pulled apart.

Close junctions could be seen also in the electrically uncoupled tissue (Figs. 6, 8, 10, 14). Because close junctions were small and infrequent, a quantitative comparison was not attempted; however, close junctions did occur between the injured cells, and they seemed to occur at least as frequently in the uncoupled tissues as in the controls.

**Electrically coupled injured tissue:** Tissue which was fixed within 1 min after injury, while at least some cells were still coupled electrically with a large injury-potential, was indistinguishable from that fixed at later times. The very dense cells had irregular outlines, the myofilaments were disarranged, and the mitochondria were swollen and clumped together. In addition, the intercellular junctions were essentially un-
FIGURE 9  Normal tissue after uranyl-acetate block stain, showing a CAP and a close junction (small arrow). The trilaminar structure of the plasma membranes and cross bridges on thick filaments are prominent. Note the dense bars (large arrows) within the CAP. The inset is a higher magnification of the marked rectangle. × 81,900; inset, × 300,500.

FIGURE 10  Electrically uncoupled injured tissue, fixed 45 min after injury. Uranyl-acetate block stain. CAPs and a close junction (small arrow) are seen. Note the dense plaque just inside the cell membrane (large arrow). The inset is a higher magnification of the marked rectangle. × 84,500; inset, × 272,000.
FIGURE 11  Control tissue, fixed immediately after injury to the other end of the trabecula. Ruthenium red exposure, unstained section. The dense ruthenium-red outlines the cells, passing through the intercellular region of the CAPs and filling the vacuoles at the cell surface. Note how CAPs 1–4 are arranged in a row along the length of the cell. The ruthenium red does not cross the cell membrane. X 12,700.

FIGURE 12  Electrically coupled injured tissue, fixed immediately after injury. Ruthenium red exposure, unstained section. The cell membranes are as impermeable to ruthenium red in this tissue, as in the controls. Note the clumped mitochondria (M) and the intact CAPs. X 14,300.
changed from the normal and control states, as was the case with the electrically uncoupled tissue. The intercellular gap width at the CAP was 290 ± 45 Å after the standard technique, and 290 ± 25 Å and 180 ± 25 Å when measured between the centers of the inner and outer leaflets of the two unit-membranes, respectively, after the tissue was stained en bloc with uranyl acetate.

Experiments with Ruthenium Red, Normal, and Control Tissue: Unstained sections showed very dense ruthenium red outlining individual cells near the surface of the tissue block (Fig. 11). Ruthenium red did not diffuse more than about 15 µ into the block, except along large connective tissue septa. It stained the external surface of the cell membrane up to and including the outer leaflet of the unit membrane (Luft, 1964; and Fig. 13) and did not pass into the cell unless the membrane was damaged (Luft, 1966). The extracellular part of the CAP was stained heavily with the ruthenium red, as was much of the rest of the intercellular space (Figs. 11, 13). In a tangential section (Fig. 16), one can see that ruthenium red did not completely fill the extracellular space of the CAP, but left globular areas unstained, giving the area a mottled appearance. Close junctions showed ruthenium red in the small gap between the two opposed cell membranes (Fig. 13). There was no junctional area between cells which could be shown to exclude ruthenium red, except the globular areas in the extracellular space between CAPs. Since no recognizable close junctions were seen in a tangential section, it is not known whether ruthenium red completely filled the gap between the opposed cell membranes or left unstained areas as in the CAP.

Experiments with Ruthenium Red, Electrically Uncoupled Injured Tissue: Tissue fixed 45 min after injury also excluded ruthenium red from intracellular structures. Both CAPs and close junctions were similar to those seen in control and electrically uncoupled tissues.

Discussion

Electrophysiology

A simplified equivalent circuit for an injury current is shown in Fig. 18. E is the difference in potential between the injured cells and the uninjured cells (if the injury causes complete depolarization, then E is the true transmembrane resting potential); r1 is the resistance of the current path in the tissue; r2 is the resistance of the current path in the external medium; and V is the measured injury potential, where

\[ V = E \frac{r_2}{r_1 + r_2} \]  

(Stampfli, 1954). A decrease in the injury potential (V) with time must be due to an increase in r1 or a decrease in E or r2. Henry et al. (1961) found in frog ventricular muscle that E does not decrease during the time that the injury potential is decreasing (Table I). The rubber diaphragm in the recording chamber insures that r2 is high for high sensitivity and does not change in any one experiment, except possibly within 1 min after the injury during the shrinkage and conformation changes of the cells. The injury potential decline with time, therefore, must be due to a gradual increase in r1.

The various resistances which make up r1 would include the resistance across the membranes of the injured cells (RM1), the resistance of the sarcoplasm of the injured cells (Rq1), the resistance of the “low resistance junctions” between the injured cells and the uninjured cells (RJ1U), the resistance of the sarcoplasm of the uninjured cells (RqU), and the resistance of the plasma membranes of the uninjured cells (RMU). RMU and RqU would not be likely to change; RqU may increase due to contracture of the myofilaments in the injured cells (see below), but this contraction takes place immediately after injury and is therefore unlikely to be the cause of the gradual increase in r1 over a 15–30 min period; RMU would be expected to decrease as a result of the injury, and, even if it recovers to its full preinjury value, this would not be enough of a resistance increase to account for the complete disappearance of the injury potential. This leaves a gradual increase in RJ1U as the most probable cause of the increase in r1, and hence the conclusion is drawn that the decrease of an injury potential with time in heart muscle is

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FIGURE 13  Control tissue, fixed immediately after injury to the other end of the trabecula, ruthenium red exposure. A CAP (large arrow) and a close junction (small arrow) are present. Inset is a higher magnification of the marked rectangle illustrating ruthenium red in the gap at the close junction and the trilaminar structure of the cell membranes. $\times 61,400$; inset, $\times 221,000$.

FIGURE 14  Electrically uncoupled injured tissue, fixed 45 min after injury, ruthenium red exposure. Several CAPs (large arrows) and a close junction (small arrow) are visible. Inset is a higher magnification of the marked rectangle. $\times 71,300$; inset, $\times 266,000$. 
FIGURE 15 Control tissue, fixed 30 min after injury to an adjacent trabecula, standard fixation. This tangential section of a CAP illustrates its mottled appearance. × 37,300.

FIGURE 16 Control tissue, fixed 45 min after injury to the other end of the trabecula, ruthenium red exposure. The tangential section of a CAP shows ruthenium red-negative areas within the extracellular space at the CAP. × 38,800.

FIGURE 17 Electrically uncoupled injured tissue, fixed 45 min after injury, ruthenium red exposure. Tangential section of a CAP shows ruthenium red-negative areas within it. × 38,000.
due to the gradual electrical uncoupling of the injured cells from the surrounding uninjured cells.

Deleze (1965, 1967) found that the cut end of a strip of mammalian cardiac muscle behaves as if a high-resistance barrier is present within a few minutes after the injury is inflicted, and that Ca++ is required for this "healing over" to occur. He suggests, without morphological evidence, that the healing over is accomplished by new membrane formation (recovery of the injured cells). It could, however, be due equally well to electrical uncoupling of the injured cells, as demonstrated in the present study.

The immediate depolarization of the injured cells is probably due to mechanical damage to the membrane at the time of injury. Continued depolarization, on the other hand, may be due to permanent loss of the membrane's selective ionic permeability or to loss of the metabolic machinery required for maintaining the Na-K pump, even if the membrane heals itself. The abnormal appearance of the mitochondria after injury is consistent with the idea that their normal biochemical functions have been disrupted (Bovis et al., 1966; Hackenbrock and Brandt, 1965; Luft and Hechter, 1957; Weinbach et al., 1967). However, there is no way to determine, from the results obtained in this study the effects of injury on membrane or mitochondrial functions, except to note that the plasma membranes are impermeable to the large ruthenium-red ions, even short times after injury.

**Electron Microscopy**

**INTERCELLULAR JUNCTIONS, CARDIAC ADHESION PLAQUES:** In mammalian cardiac muscle, two morphologically distinct intercellular junctions can be observed, (a) desmosomes and (b) myofibrillar insertion plaques (Muir, 1965, 1967) or interfibrillar portions of the intercalated disc (Sjöstrand et al. 1958). The cardiac adhesion plaque (CAP) appears to be analogous to both types of junction. CAPs oriented parallel to the fiber direction are referred to as desmosomes by Barr et al. (1965, frog atrium); Fawcett and Selby (1958, turtle atrium); Grimley and Edwards (1960, toad ventricle); Karrer and Cox (1960, mouse pulmonary vein); and Staley and Benson (1968, frog ventricle). Those CAPs perpendicular to the fiber direction are called intercalated discs by Fawcett and Selby (1958, turtle atrium); Grimley and Edwards (1960, toad ventricle); Karrer

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**TABLE I**

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<th>The Change of Measured Injury Potentials and Intracellular Potentials with Time after Injury in Frog Ventricular Muscle*</th>
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*From Henry et al., 1961.
and Cox (1960, mouse pulmonary vein), and Staley and Benson (1968, frog ventricle). Since these junctions appear to differ primarily in their orientation with respect to the fiber direction, a single, common term can refer to all such junctions, at least in the above-mentioned tissues. This type of junction is morphologically distinct from the typical epithelial desmosomes as described by Farquhar and Palade (1965). In addition, these junctions are found in other places besides the myofilament insertions, and they bear little resemblance to the complex structures in mammalian heart muscle which were originally termed intercalated discs by the light microscopists. It was felt, therefore, that the term cardiac adhesion plaque (CAP) would be a useful designation for the type of junction seen in this tissue, in order to avoid the prejudicial connotations inherent in the other above-mentioned terms.

The failure of ruthenium red to fill uniformly the intercellular gap at a CAP deserves comment. This suggests that there may be areas within the junction which are inaccessible to extracellular ions, or at least to cations as large and as heavily charged as ruthenium red. The CAPs of frog ventricular muscle have the same mottled appearance when stained with ruthenium red when viewed in a tangential section (Baldwin, unpublished results).

Kelly (1966), after describing ruthenium red-stained desmosomes in newt epidermis, suggests that there may be pillars of material extending across the intercellular space which are continuous with the middle (lucent) layer of the unit membranes of the two adjacent cells. If this is true, similar ruthenium red-negative areas might also be found in desmosomes as well as in CAPs. However, Kelly's technique is such that he was demonstrating ruthenium red-positive material (very light staining with ruthenium red); the results reported here only distinguish ruthenium red-negative areas (rather heavy staining, with ruthenium red-positive material filling the extracellular fluid space). Consequently, an exact correlation between the two studies cannot be made.

Close junctions: Until recently, all intercellular junctions which were characterized by close apposition of the membranes of the two adjoining cells were referred to as tight junctions or zonulae occludentes (Farquhar and Palade, 1963, 1964). Revel and Karnovsky (1967) have shown, using a very fine lanthanum oxide sol as an extracellular tracer, that those junctions which previously were described as tight junctions should be separated into two groups; the true tight junctions or zonulae occludentes; and the close or gap junctions. The former type is impermeable to lanthanum (Revel and Karnovsky, 1967; Brightman and Reese, 1969), ruthenium red (Martinez-Palomo, 1968) and horseradish peroxidase (Brightman and Reese, 1967, 1969) and can be considered to be a true membrane fusion (Farquhar and Palade, 1963, 1965). The latter type of junction is permeable to the lanthanum sol (Revel and Karnovsky, 1967), ruthenium red (Martinez-Palomo, 1968), and horseradish peroxidase (Brightman and Reese, 1967, 1968, 1969). Using these extracellular tracers or a uranyl-acetate block stain, a gap of approximately 20 A can be demonstrated between the outer leaflets of the two opposed cell membranes (Revel and Karnovsky, 1967; Brightman and Reese, 1967, 1969).

The present results show that the close membrane appositions of frog atrium are close, or gap, junctions. If such close junctions were cut in a tangential section, areas within the extracellular space of the junction might not be penetrated by the ruthenium red. However, close junctions are so small, so infrequent, and so randomly distributed that there is no way to recognize with confidence a close junction except when it is cut perpendicularly.

In contrast to these results, Barr et al. (1965) have reported tight junctions (nexuses) in frog atrium after permanganate fixation. Brightman and Reese (1967, 1969) present evidence that permanganate fixation transforms gap (close) junctions into the tight variety in central nervous tissue. It is possible that a similar transformation occurs in cardiac tissue as well, which would explain the discrepancy between the results of Barr et al. and those of the present study.

Although large areas of close membrane apposition can be found in mammalian ventricular muscle (Barr et al., 1965; Karrer, 1960; Muir, 1965, 1967; Sjöstrand et al., 1958; Sjöstrand and Anderson-Cedergren, 1960; Sommer and Johnson, 1968; Fawcett, 1965), it appears that this is not true of all types of cardiac muscle. A paucity of such junctions has been reported for frog ventricular muscle (Staley and Benson, 1968; Som-

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mer, 1968), for chicken ventricular muscle (Sommer, 1968; Scott, 1969),4 and for Purkinje fibers of a variety of mammals (Sommer and Johnson, 1968). In addition, James and Sherf (1968) have been unable to find close membrane contacts between P (Pacemaker?) cells in dog and human hearts. It has been claimed that a lack of close membrane appositions in frog atrium is a result of shrinkage during tissue preparation (Barr et al., 1965; Dewey and Barr, 1964), but in the results reported here there is no evidence of such shrinkage in the control tissue. Furthermore, close junctions still remain intact, even when the cells shrink in contracture after injury (see below), and Cobb and Bennett (1969) have reported that nexuses between smooth muscle cells of taenia coli, vas deferens, and gizzard were unaffected by shrinking the cells in hypertonic solutions. Moreover, variations in amounts of close membrane appositions can be seen in different tissues of the same heart. For example, in chicken heart, there are many such areas between Purkinje cells, but very few between ventricular cells (Sommer, 1968; Scott, 1969).4 It therefore seems unlikely that differences in amounts of close membrane appositions can be ascribed solely to differences in fixation or processing.

The few observations of relatively large areas of close junction in association with abnormal mitochondria and/or sarcoplasmic reticulum (Fig. 19) may be interpreted as additional evidence that such large areas of close junction are not normally present in frog atrium. It is quite possible that the same conditions of tissue preparation or fixation which result in abnormal mitochondria and sarcoplasmic reticulum also result in abnormal intercellular relationships. It is improbable that conditions of fixation which are poor for preserving mitochondria and sarcoplasmic reticulum are very good for preserving intercellular relationships. Brightman and Reese (1969) have recently reported on finding “labile appositions of cell membranes” in central nervous tissue which they believe to be fixation artifacts. These labile appositions are particularly prevalent in tissue which shows signs of inadequate preservation (i.e., dilated cisternae of endoplasmic reticulum), and therefore may be similar to the relatively large areas of close junction seen in frog atrium.

**Changes with Injury:** The morphological changes which take place at the intercellular junctions after injury are minor. Although CAPs which have been pulled apart are observed somewhat more frequently in experimental than in control tissue, still their presence is exceptional. Within the CAP itself only two differences are observed after injury: a dense plaque appears close inside the cell membrane, and the linear density which is located in the center of the intercellular space becomes more apparent. The appearance of the dense plaque and linear density may be due to an orientating mechanical effect of the pull of the strongly contracted myofilaments. Another possibility is that they are normally present, but masked by other dense materials which relocate after injury. The first possibility, that of mechanical alterations, is unlikely to account for the intercellular linear density because the spacing between the cells at the CAPs does not change appreciably after injury.

The occurrence of close junctions in the injured tissue would indicate that these junctions are not disrupted after injury, or at least that most remain intact. Unlike the case with CAPs, it would be difficult to tell where a close junction had been located after it had been pulled apart, since the cytoplasmic modifications at this type of junction are minimal. Comparing the frequency of occurrence of these junctions in control and experimental tissue, the conclusion is reached that they do in fact remain intact, but their infrequent occurrence makes a good quantitative comparison difficult.

The most striking changes after injury are an increase in cytoplasmic density and a disarrangement of the myofilaments. This latter change is probably due to contracture, since a slow, sustained contraction of an in situ injured area can be observed. This contraction is complete within 1 min of inflicting the injury. When a banding pattern can be detected in an injured cell (Fig. 12), the repeat distance is very short, 0.5-0.6 μ, as compared to the normal length of the A band, 1.5 μ. The increase in density of the cytoplasm may be due in part to water loss and shrinkage, as suggested by the scalloped appearance of the injured cell surfaces and by the decrease in spacing between the myofilaments.

The techniques used in this project make it impossible to know if a given pair of cells seen in the

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It seems reasonable, however, to assume that injured cells seen in the electrically uncoupled tissues are in fact electrically uncoupled from their surrounding cells as most cells in such tissues must be electrically isolated. At the very least, these cells must be typical of such uncoupled cells since they were typical of the injured cells which were uniformly and consistently observed in uncoupled tissues. It is thought that even the infrequent close junctions are likely to be present between electrically uncoupled cells. There was no obvious decrease in their frequency in the electrically uncoupled tissues as surely would be the case if close junctions came apart when cells became electrically isolated.

In the electrically coupled injured tissues it is less tenable that the injured cells observed were in fact electrically coupled. One knows only that enough injured cells are electrically coupled to uninjured cells to give rise to the injury current. However, it seems likely that cells such as those shown in Fig. 12 are typical of electrically coupled injured cells since they were typical of the whole mass of cells in the electrically coupled tissues.

Electrical Coupling between Cells

Electrotonic interactions can be found between a wide variety of cells (Barr et al., 1965, 1968; Barr and Berger, 1964; Loewenstein, 1966; Loewenstein and Kanno, 1964; Loewenstein et al., 1965; Loewenstein and Penn, 1967; Penn, 1966; Tillie, 1966; Potter et al., 1966; Woodbury and Crill, 1961; Woodbury and Gordon, 1963; Bennett et al., 1963; Pappas and Bennett, 1966; Revel and Sheridan, 1963; Rosenbluth, 1965; Sheridan, 1966). The mechanism of electrical coupling be-
tween cells is unknown, but presumably there exists some sort of intercellular junction with low electrical-resistance properties. It is reasonable, but unproven, to assume that the physiological junction is coincident with an anatomical junction.

Two types of intercellular junctions have been proposed to be electrical couplings because of their frequent occurrence between electrically coupled cells: (a) septate junctions of invertebrates (Wood, 1959), which are present between salivary gland cells of insects, have been suggested as the sites of electrical coupling in this tissue (Loewenstein and Kanno, 1964); (b) "tight" junctions are often found between electrically coupled vertebrate cells, and this type of junction has been proposed as being involved in electrical communication by Bennett et al. (1963), Robertson (1961, 1963), Dewey and Barr (1962, 1964), Weidmann (1965, 1966), Farquhar and Palade (1964, 1965), Loewenstein et al. (1965), Penn (1966), Potter et al. (1966), Revel and Karnovsky (1967), and Revel and Sheridan (1968). However, in many cases it is not known whether these tight junctions are true tight junctions or are gap or close junctions since the gap junction was first described after many of these references were published. For the purpose of convenience and clarity in this discussion, all such junctions will be referred to as tight junctions since this term has been so commonly used in the literature.

Tight and septate junctions are not the only type of intercellular junction found between electrically coupled cells, but they do occur more regularly than desmosomes, intermediate junctions, CAPs, etc. However, there are electrically coupled tissues in which tight junctions either have not been observed or, if present, are few and small. Such is the case with some types of cardiac muscle, as has been discussed above. Choi (1963) was unable to find any intercellular junctions between smooth muscle cells in toad bladder after osmium tetroxide and permanganate fixatives, and Barr et al. (1968) found tight junctions between intestinal smooth muscle cells only after the cells were incubated in Krebs-Henseleit solution for several hours prior to fixation. Thus it would appear that, even if tight junctions are sites of electrical transmission between cells, they may not be the only sites of low resistance. Similar conclusions were reached by Rosenbluth (1965), and Martin and Veale (1967).

A further characteristic of tight and septate junctions is that they are not sufficient in themselves to assure low electrical-resistance between cells. On one hand, Barr et al. (1965) uncoupled frog atrial cells in hypertonic sucrose and found that the nexal areas (close junctions?) separated under this treatment, and Pappas (1968) found that the tight junctions which were present between nerve cells in the septate axon of crayfish are separated when the cells are electrically uncoupled. On the other hand, Bullivant and Loewenstein (1968) found that there was no morphologically detectable change in the septate junctions when salivary gland cells are uncoupled. And in the present study, close junctions remained intact even when the cells were electrically uncoupled. Thus it seems that, although physical separation of membranes at tight junctions will cause uncoupling, separation need not occur to get the same effect, and septate junctions can be uncoupled when they appear morphologically intact.

Loewenstein and coworkers (Loewenstein, 1966, 1968; Loewenstein et al., 1967; Loewenstein and Penn, 1967) account for the properties of the low-resistance pathways between cells by proposing that plasma membranes have low electrical-resistance when exposed to low Ca++ solutions on both sides of the membrane. They assume that electrical coupling depends upon the structural integrity of the plasma membrane in excluding Ca++ from the internal surfaces of the junctional membranes, and on the structural integrity of the junctional area in excluding Ca++ from the external surfaces of the cell membranes at the junction. Uncoupling of the cells, then, is due to Ca++ gaining access to the junctional membranes and in some way making them impermeable to ions. Uncoupling with injury (as in this study) would therefore result from Ca++ gaining access to the inside of the cell through the damaged membrane and sealing off the junctional membrane from the inside of the cell (Loewenstein et al., 1967; Loewenstein and Penn, 1967).

The pattern of distribution of ruthenium red around cells is of interest in light of the hypothesis that low Ca++ concentration is responsible for low-resistance membranes at cell junctions. Ruthenium red has a molecular weight of 858.5 and a charge of +6 (Luft, 1965). It is therefore reasonable to assume that Ca++ can diffuse into any intercellular space in which ruthenium red is found, although it is not necessarily true that an

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absence of ruthenium red means an absence of Ca++. Since the theory proposed by Loewenstein's group calls for intercellular junctions which exclude Ca++ from the external surfaces of the cell membrane at these junctions, any such junctions would be expected to exclude ruthenium red also. In the results of this study, the only possible areas which fit this requirement are the small (300 A in diameter) areas seen within tangentially cut CAPs, and perhaps small areas within the close junctions. Although it is not necessarily true that areas which are ruthenium red-negative also exclude Ca++, this is a possibility. If this is true, then these small areas may be sites of low membrane-resistance and thus of electrical coupling between cells. Although each of these ruthenium red-negative areas is small, there are many within each CAP (approximately one-fourth the total area of the CAP is ruthenium red-negative), so that their total area on a particular cell might be quite large. There is no change in these ruthenium red-negative areas after injury. However, if the junction is sealed off from the inside of the cell membrane as proposed by Loewenstein et al. (1967) and Loewenstein and Penn (1967), then one would not necessarily expect to see a change here.

The evidence that Ca++ is involved in the mechanism of electrical coupling of cells is well established (Loewenstein, 1966, 1968; Loewenstein et al., 1967; Deleze, 1967; and de Mello et al., 1969), but it is difficult to know just how its effects are mediated. Even though Ca++ is known to affect membrane permeability to ions (Weidmann, 1955; Caputo and Gimenez, 1967; and Curtis, 1963), it is also known to affect intercellular adhesion (de Mello, 1969; Muir, 1967; and Sedar and Forte, 1964) as well as take part in a variety of metabolic reactions. Thus Ca++ may not be directly affecting cell membranes as proposed by Loewenstein, but instead affecting intercellular communication in some indirect way.

It is interesting to note that Jochim et al. (1935) found that they could maintain injury potentials in heart for long periods of time (the injured cells did not uncouple) by injuring the cells with a slight pressure on the recording electrode itself. Similar effects have been obtained with suction electrodes (Churney and Ohshima, 1964; Sjöstrand, 1966). These long-lasting injury potentials can be explained by assuming that the injury is not so great that the metabolic activity of the cell is destroyed, and that the injured region is confined to the inside of the electrode. If such conditions were met, then no Ca++ could enter the cell to cause uncoupling.

Conclusions

Any changes in intercellular relationships which occur when cells uncouple are beyond the resolution of the techniques used in this study. However, the results are incompatible with the idea that close membrane appositions per se will insure electrical coupling between cells.

This investigation was supported by Predoctoral Fellowship Grant Nos. 1-F1-GM-30, 051-01; 5-F1-GM-30,051-02; 5-F1-GM-30,051-03; and 4-F1-GM-30,051-04 from the United States Public Health Service. Preparation of this paper was undertaken while the author was under the sponsorship of a Science Research Council Grant to Professor A. R. Muir at the Royal (Dick) School of Veterinary Studies, Edinburgh, Scotland. Publication costs were met by United States Public Health Service Grants GM-00136 and GM-16598.

The author wishes to express her appreciation and gratitude to Dr. John H. Luft for his invaluable encouragement and advice during this investigation. She would also like to thank Dr. Luft and Dr. A. R. Muir for their critical reading of the manuscript, Dr. J. W. Woodbury for his assistance and advice, and Miss Christine Aitken for typing the manuscript. Received for publication 28 July 1969, and in revised form 13 March 1970.

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