Propagation of Action Potentials and the Structure of the Nexus in Cardiac Muscle

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ABSTRACT The hypothesis that the nexus is a specialized structure allowing current flow between cell interiors is corroborated by concomitant structural changes of the nexus and changes of electrical coupling between cells due to soaking in solutions of abnormal tonicity. Fusiform frog atrial fibers are interconnected by nexuses. The nexuses, desmosomes, and regions of myofibrillar attachment of this muscle are not associated in a manner similar to intercalated discs of guinea pig cardiac muscle. Indeed, nexuses occur wherever cell membranes are closely apposed. Action potentials of frog atrial bundles detected extracellularly across a sucrose gap change from monophasic to diphasic when the gap is shunted by a resistor. This indicates that action potentials are transmitted across the gap when sufficient excitatory current is allowed to flow across the gap. When the sucrose solution in the gap is made hypertonic, propagation past the gap is blocked and the resistance between the cells in the gap increases. Electron micrographs demonstrate that the nexuses of frog atrium and guinea pig ventricle are ruptured by hypertonic solutions.

Transmission of action potentials from one cardiac cell to another is apparently due to electrotonic spread of current. There is extensive electrotonus along frog atrial fibers (Trautwein, Kuffler, and Edwards, 1956) as well as along sheep Purkinje fibers (Weidmann, 1952), rat atrium (Woodbury and Crill, 1961), and strands of cultured avian atrial cells (Sperelakis and Lehmkuhl, 1964). Action potentials of frog atrial fibers will propagate past a sucrose gap when an appropriate shunt resistor allows current flow between the extracellular electrolyte solutions (Barr and Berger, 1964). Currents generated by atrial fibers, themselves, are sufficient to allow propagation in the frog atrium.

Some portion of the cell surfaces must be specialized to allow appreciable current flow between cell interiors. In fact, electron micrographic studies have demonstrated regions between cardiac muscle cells where the opposing cell membranes are fused along their outer lamellae. Such regions have been called nexuses. Furthermore, it has been suggested that nexuses are sites of

electrotonic coupling between adjacent cardiac and smooth muscle cells (Dewey and Barr, 1962; Dewey and Barr, 1964). Similar, if not identical, regions have been demonstrated at certain electrical synapses (Bennett, Aljure, Nakajima, and Pappas, 1963; Dewey and Barr, 1964; Robertson, 1961, 1963), between neurons and glial cells (Dewey, unpublished), between glial cells (Gray, 1961; Peters, 1962), and in various epithelia (Dewey and Barr, 1964; Farquhar and Palade, 1963; Robertson, 1960; Sjöstrand, 1960). In contrast to the occurrence of nexuses at electrical synapses there is a synaptic cleft at chemical synapses (Eccles, 1964). Recently there have been discussions of the theoretical advantages of a synaptic cleft for chemical transmission (Eccles and Jaeger, 1958) and the nexus for electrical transmission between cells (Barr, 1963; Dewey and Barr, 1964). Electrotonic coupling between cells would be favored by a low resistance pathway for current flow between the

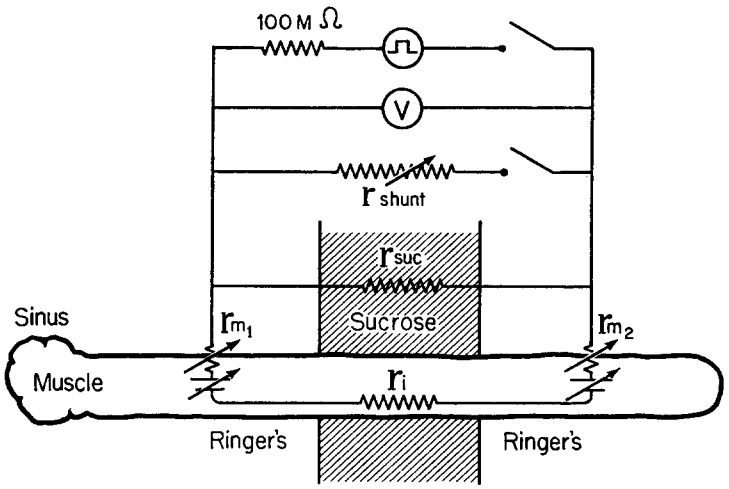

FIGURE 1. A schematic representation of the experimental arrangement.

cell interiors. Three conditions which usually lead to low membrane resistance are present at the nexus. They are: (*a*) nexal membranes separate similar solutions so that there can be no diffusion potential across the nexus; (*b*) nexal membranes are exposed only to high potassium solutions; and (*c*) they are exposed only to low calcium solutions.

The purpose of this paper is to elucidate the effects of solutions of different tonicity on the structure of the nexus and on the electrical properties of cardiac muscle. It is shown that the cells are decoupled electrically when the nexuses come apart. This corroborates the hypothesis that the site of electrotonic coupling is the nexus.

METHODS

Frog atrial bundles were used as the primary experimental material because they are easily studied with the sucrose gap technique. The structural effects of hypertonic

solutions on guinea pig papillary muscle were investigated also. This was done because the anatomical relationships between frog atrial cells are different from those at the intercalated disc of mammalian cardiac muscle.

Electrical Recording

In the frog atrium bundles of fibers form a complex network which makes up the major portion of the atrial wall. Some bundles run as trabeculae for several millimeters. Such bundles from *Rana esculenta* or *temporaria* were used in the electrical experiments. A piece of sinus venosus or atrioventricular node was left at one end of the bundle to provide spontaneous pacemaker activity.

The experimental arrangement is shown schematically in Fig. 1. A modified sucrose gap (Stämpfli, 1954) was used. In the gap region sucrose replaces the ions of the extracellular space, thereby dividing the extracellular compartment into three regions along the bundle. Rubber membranes, 70 μ in thickness, separated the flowing sucrose solution from Ringer's solutions. The gap was from 400 to 600 μ wide (Barr and Berger, 1964; Berger, 1963).

Electron Microscopy

FROG ATRIAL MUSCLE

Hearts were removed from decapitated, spinally pithed frogs. Frog atria (*Rana pipiens*) were isolated and opened from the dorsal aspect along the interatrial septum. A group of from 4 to 10 trabeculae of atrial muscle was dissected from the ventral wall of the atrium and tied at either end to a small piece of balsa wood. Occasionally dissections were made to include sino-atrial node to insure continued pacemaker activity within the preparations during subsequent treatment. Such activity was observed with the aid of a dissecting microscope.

Tied atrial strips were incubated in the media described below and then fixed for varying lengths of time with permanganate (MnO_4^-) fixatives. Fixation times ranged from 30 minutes to 4 hours. Cytoplasmic structures such as myofibrils were overfixed to the point of disaggregation by fixation times longer than 30 minutes whereas the outer lamellae of the membranes were somewhat better seen in specimens fixed for longer times.

All dissections were performed in normal Ringer's solution (Prosser and Brown, 1961). The tied muscle bundles were transferred to fresh Ringer's solution at room temperature for 15 minutes to 1 hour. Some specimens were incubated in sucrose solutions of either 110, 230, or 670 mOsM for a period of 10 minutes. Others were incubated in solutions of equal osmolarity made by adding sodium chloride or sucrose to normal Ringer's solution. They were then transferred into fixatives of the corresponding osmolarity. Control specimens were incubated for the 10 minute period in fresh Ringer's solution. Some preparations were made by performing the dissection, incubation, and fixation at 4°C but this did not give different results.

GUINEA PIG PAPILLARY MUSCLE

Guinea pigs were killed by a blow at the base of the skull, and the hearts quickly removed and thoroughly rinsed in Krebs-Henseleit (K-H) solution (Krebs and Hense-

leit, 1932). The atria were removed and an incision made along the posterior wall of the right ventricle. The septal papillary muscle was freed from the septal cusp of the tricuspid valve and excised at its base. The papillary muscle was placed immediately in fresh K-H solution at 37°C to allow recovery.

The papillary muscles were then placed in various solutions for an incubation period of 30 minutes at which time they were transferred into appropriate fixatives (see below). The incubating solutions were of three types: (*a*) hypoosmotic, *i.e.* K-H solution diluted with an equal volume of distilled water; (*b*) isosmotic, *i.e.* normal K-H solution; and (*c*) hyperosmotic, *i.e.* K-H to which was added 145 gm of sucrose per liter. The final osmolarities were 160 mOsM, 310 mOsM, and 630 mOsM, respectively. The effects of incubation in hyperosmotic solutions were reversed by returning specimens to isosmotic solutions for 30 minutes before fixation. All specimens were fixed at the same osmolarity as the incubation solution.

FIXATION AND SUBSEQUENT TREATMENT

The composition of the fixatives varied depending on the final osmolarity desired. The osmolarity of all fixatives was measured on an Advanced Instruments osmometer model 65-31 and was expressed as sodium chloride equivalents. The pH of all fixatives was 7.4.

The MnO_4^- fixatives contained 0.5 per cent or 1.0 per cent sodium permanganate buffered with 0.04 M veronal-acetate. Hypoosmotic fixative was always 0.5 per cent sodium permanganate. Sodium chloride was used to adjust the osmolarity of the hyperosmotic fixatives. The final osmolarities of the fixatives were measured to be: (*a*) hypoosmotic, 160 mOsM; (*b*) isosmotic, 320 mOsM; and (*c*) hyperosmotic, 630 mOsM for fixation of guinea pig muscle and (*a*) hypoosmotic, 127 mOsM; (*b*) isosmotic, 230 mOsM; and (*c*) hyperosmotic, 670 mOsM, for fixatives used on frog atria.

In addition to MnO_4^- fixatives, osmic acid (OsO_4) and glutaraldehyde with postosmication were employed. The OsO_4 fixatives contained 1.0 per cent of OsO_4 and 0.028 M veronal-acetate. The osmolarities were made equal to those of the permanganate fixatives by the addition of sucrose. A 2.5 per cent w/v solution of glutaraldehyde in 0.09 M sodium phosphate buffer (630 mOsM) was used to fix specimens for 2 hours at which time they were washed in Ringer's solution for 18 hours and then were fixed for 2 hours in the isosmotic OsO_4 fixative described above.

All specimens were dehydrated rapidly in a graded series of ethyl alcohols and embedded in epon 812. Sections were obtained using an LKB Ultrotome and mounted on carbon-coated formvar films. Sections were stained for 18 hours in 2.0 per cent uranyl acetate and for 10 minutes by the lead procedure described by Karnovsky (1961). Electron photomicrographs were obtained on a Hitachi HU-11A electron microscope.

MEASUREMENT OF FROG ATRIAL FIBER LENGTH

Three or four atrial bundles were dissected from the ventral wall. These bundles were shaken in calcium-free Ringer's solution to which had been added 0.02 per cent EDTA or 0.01 per cent trypsin or both. Incubations from one-half to 2 hours usually yielded preparations which could be teased apart so that single, isolated cells were ob-

tained. Isolated cells were photographed and measurements of cell and sarcomeric lengths were made directly from photographic enlargements.

FERROCYANIDE SPACE OF GUINEA PIG AND FROG CARDIAC MUSCLE

Papillary muscles from guinea pig heart were incubated in a modified solution (355 mOsM) which contained 9 gm sodium ferrocyanide per liter replacing the sodium chloride. Frog atria were incubated for one-half hour in a modified Ringer's solution (238 mOsM) containing 34.6 gm sodium ferrocyanide/liter as a replacement for sodium chloride.

Following incubation in the ferrocyanide solutions, specimens were fixed in a solution containing 3.6 per cent w/v formaldehyde, 1.2 per cent ferric ammonium sulfate, and 0.38 per cent w/v HCl, for 2 hours (Allen, 1955). They were dehydrated rapidly

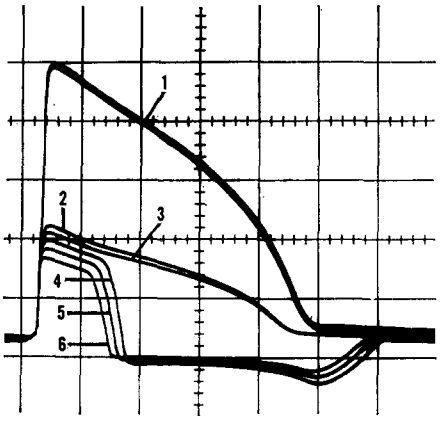

FIGURE 2. Records of frog atrial action potentials recorded with the arrangement shown in Fig. 1. Amplitude and waveform are a function of shunt resistance. Propagation of action potential across the sucrose gap is indicated by the change from monophasic to diphasic action potential. Curves 1–6 are with r_{sh} infinity, 10^5, 9×10^4, 8×10^4, 7×10^4, and 6×10^4 ohms, respectively. Divisions mark 20 mv and 100 msec. Gap width 400 μ.

and double embedded in celloidin and paraffin. Sections 4 μ in thickness were obtained, cleared, and mounted directly without further staining.

RESULTS

Electrical Recording

The action potentials that can be recorded from frog atrial bundles using the sucrose gap compare favorably with those obtained using microelectrodes (Brady, 1964; Trautwein, Kuffler, and Edwards, 1956; Van der Kloot and Dane, 1964). Curve 1 in Fig. 2 is the action potential of an atrial bundle. If the equivalent circuit shown in Fig. 1 is a useful approximation of the actual experimental situation, then:

$$AP_{obs} = APr_{sh}r_{suc}/[r_{sh}r_{suc} + (r_{sh} + r_{suc})(r_i + r_{m_1} + r_{m_2})] \qquad (1)$$

holds. In equation (1), AP_{obs} is the observed amplitude of an action potential of true amplitude AP, r_{sh} is the resistance of the external shunt pathway, r_{suc}

is the effective shunt resistance through the sucrose compartment, r_i is the effective longitudinal resistance from the cell membranes on one side of the gap to the cell membranes on the other side, and finally, r_{m_1} and r_{m_2} are the effective resistances of the cell membranes in the two Ringer pools. For the observed amplitude AP_{obs} to approach the true amplitude, r_{suc} must be much larger than $(r_i + r_{m_1} + r_{m_2})$ (see Fig. 2, curve 1).

Equation (1) can be rearranged to give:

$$1/AP_{obs} = [1 + (r_i + r_{m_1} + r_{m_2})(1/r_{suc} + 1/r_{sh})]/AP \qquad (2)$$

Comparison of curves 1 and 2 of Fig. 2 shows that as the shunting resistance was decreased down to some minimum value, the shape of the observed monophasic action potential was changed very little. It was simply attenuated in-

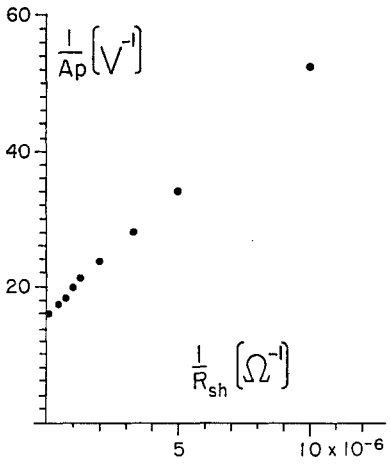

FIGURE 3. The reciprocal of the amplitude of the recorded frog atrial action potential is shown to be a linear function of the reciprocal of the shunt resistance in accordance with equation (2).

dicating that propagation across the gap had not occurred. Fig. 3 shows, as expected from equation (2), that the reciprocal of the amplitude of the observed action potential is linearly related to the reciprocal of the shunt resistance.

The excitatory current through the segment in the gap is limited by the longitudinal resistance plus the resistance of the shunt. It is probably for this reason that narrow gaps were necessary for propagation past the sucrose gap. When the gap was narrow enough and the shunt resistance was decreased enough to allow current sufficient for propagation, the action potential became diphasic in an all-or-none manner. Fig. 2 shows that the lower the shunt resistance the earlier the action potential jumped across the sucrose gap.

Using the hyperosmotic sucrose solution in the gap instead of the isosmotic sucrose solution caused an increase in the longitudinal resistance of the portion of the bundle in the gap. This increase of resistance was detected by applying constant current pulses as indicated in Fig. 1 and observing the increased

voltage of the pulses (Fig. 4). From Fig. 1 it can be deduced that:

$$V_p = I_p(r_i + r_{m_1} + r_{m_2})r_{sh}r_{suc}/[r_{sh}r_{suc} + (r_{sh} + r_{suc})(r_i + r_{m_1} + r_{m_2})] \quad (3)$$

where I_p is the pulse current and V_p is the observed pulse voltage. By combining equations (1) and (3) the change of the longitudinal resistance with sucrose osmolarity can be estimated. The longitudinal resistance term always has with it the membrane resistance terms. That the latter are relatively smaller is seen by the fact that the pulse height was constant through the action potential and when KCl was applied to the post-gap Ringer pool. Also the length constant for these fibers is small (Trautwein, Kuffler, and Edwards,

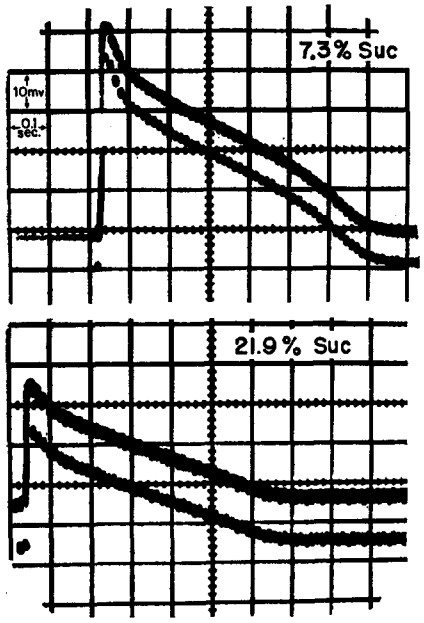

FIGURE 4. Monophasic action potentials from frog atrium recorded while 8 msec. rectangular pulses of 5 ma were passed through the preparation. Upper record was obtained with an isosmotic sucrose solution in the gap while the lower record was obtained with a hypertonic sucrose solution in the gap. The decreased amplitude of the action potential and the increased pulse height signal the increase of longitudinal resistance. Gap width 600 μ.

1956; Van der Kloot and Dane, 1964). It is assumed here for convenience that $r_i \gg (r_{m_1} + r_{m_2})$ without further proof. By combining equations (1) and (3),

$$r_i(\text{hyper})/r_i(\text{iso}) = AP_{obs}(\text{iso}) \cdot V_p(\text{hyper})/AP_{obs}(\text{hyper}) \cdot V_p(\text{iso}) \quad (4)$$

is obtained. The relative change in longitudinal resistance with changed sucrose osmolarity can be estimated.

Fig. 4 shows two monophasic action potentials upon which were superimposed voltage pulses due to constant current pulses. One action potential was recorded with an isosmotic sucrose solution in the gap and the other was recorded with a hyperosmotic sucrose solution in the gap. The longitudinal resistance changed by a factor of at least 2.7. The increase of resistance was reversible upon reintroduction of isosmotic sucrose solution into the gap. Other

experiments gave changes as large or larger, although in many the propagation failed with a smaller change of the longitudinal resistance.

The increase of longitudinal resistance of the portion of the bundle in the hypertonic sucrose can prevent the usual propagation of action potentials past the gap when the shunt resistor is switched into the circuit. As seen in Fig. 5, the switching in of the shunt resistor for every second action potential yielded alternate monophasic and biphasic action potentials. That the latter represented the spread of activity over the entire postgap portion of the bundle was indicated by the normal contraction of that part of the bundle.

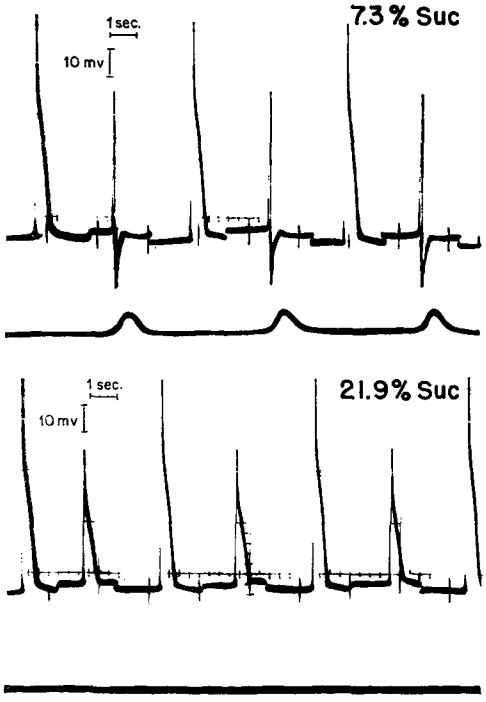

FIGURE 5. Isosmotic sucrose solution was in the gap when upper record was obtained. In this record of frog atrial activity the upper trace shows alternating monophasic and diphasic action potentials according to whether the shunt resistance was absent or present. Lower trace shows contraction of the postgap muscle when the action potential is propagated past the gap.

In the lower record, the upper trace shows only monophasic action potentials when hypertonic sucrose was in the gap. The lower trace shows that when the propagation of the action potential was blocked, no mechanical activity was seen beyond the gap. Gap width 400 μ.

When a hyperosmotic sucrose solution was substituted for the isosmotic solution, the propagation past the gap by means of the shunt was stopped. The longitudinal resistance increased so much after 10 or 15 minutes that even a direct short (2200 ohms) across the gap failed to allow propagation past the gap. The complete blocking of propagation due to hypertonic sucrose solutions in the gap was reversible and the whole experiment was performed repeatedly on the same preparation.

The converse experiment gave the expected results. When the isosmotic sucrose solution flowing through the gap was replaced by a hypoosmotic sucrose solution, propagation past the gap was facilitated. Fig. 6 shows that a resistance too high to allow propagation with an isosmotic sucrose gap was

adequate after a hypoosmotic sucrose solution had flowed for less than 15 seconds. Again the experiment was readily reversible.

The equivalent experiments using solutions of 3, 2.5, 2, 1.5, 0.75, and 0.5 times the isosmotic sucrose concentration could be performed on the same preparation. All the hyperosmotic solutions caused blockage of propagation even with a direct short across the gap. The time for this to occur decreased

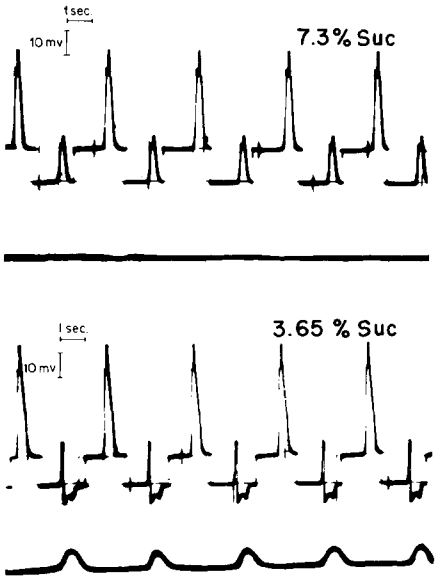

FIGURE 6. Isosmotic sucrose solution was in the gap when the upper record of frog atrial activity was obtained. The shunt resistance was set a little larger than that allowing propagation. The jump of the upper trace when the shunt was switched in was due to imbalance across the sucrose gap. The upper trace shows only monophasic action potentials and the lower trace shows no postgap contraction.

In the lower record, about 10 seconds later, diphasic action potentials on the upper trace indicate that propagation occurred when an isosmotic sucrose solution was replaced by a hypoosmotic one. Lower trace shows mechanical activity paralleled the action potentials. Gap width 400 μ.

from about 2 minutes for twice normal to about 5 to 15 seconds as the sucrose concentration increased to three times normal. The 0.75 normal sucrose solutions caused no obvious change in propagation.

Structure of Amphibian Atrial Muscle

The gross arrangement of muscle cells in amphibian atria is strikingly different from that in mammalian atria. The muscle cells or fibers are arranged in fasciculi or bundles as illustrated in Figs. 7 and 9. Each bundle contains on the order of a hundred cells in parallel. Bundles surrounded by an endocardium form trabeculae. Those bundles lying in the atrial wall are covered partially


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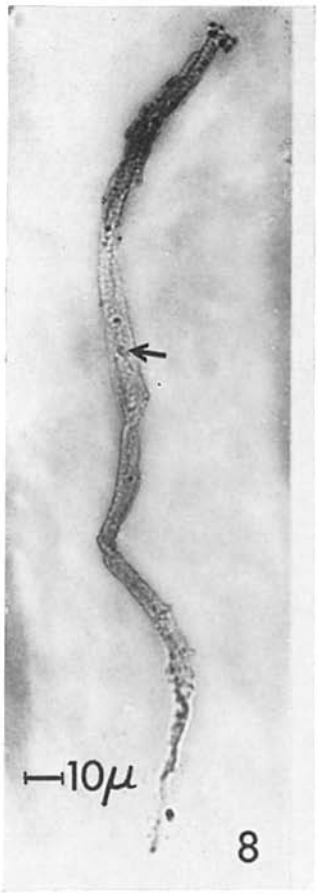
7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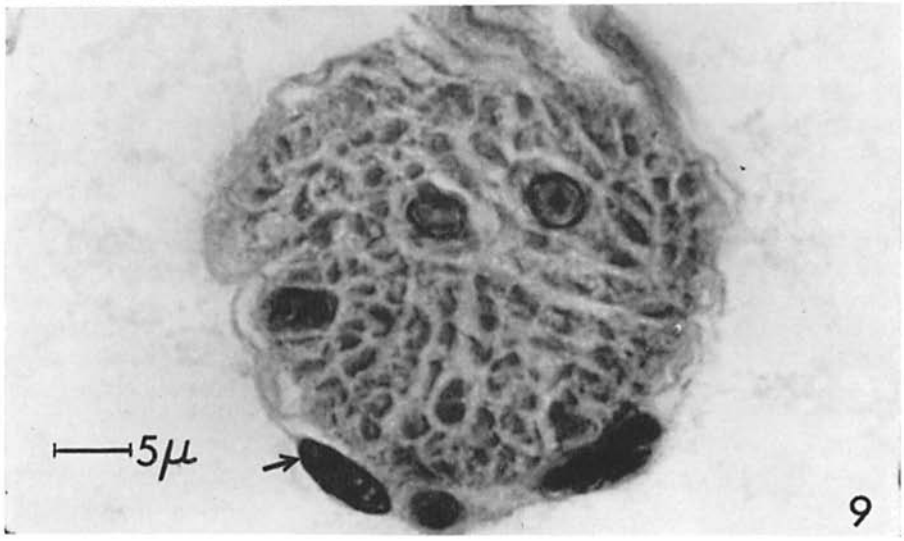
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by endocardium. The endocardium consists of a simple squamous epithelium and a thin underlying layer of connective tissue fibers; *i.e.*, collagen. Between the collagen fibers and the underlying muscle cells is a typical basement membrane. The basement membrane and associated collagen penetrate between groups of muscle cells subdividing the bundle. Thus, while non-nodal mammalian cardiac fibers are ensheathed by a basement membrane individually, frog atrial muscle cells lie adjacent to each other without intervening basement membrane.

The individual muscle cells of frog atrium are shaped differently from mammalian cardiac muscle cells. The latter are cylindrical with flattened interdigitating ends at the intercalated disc (Sjöstrand, Andersson-Cedergren, and Dewey, 1958). Frog atrial fibers are long tapering cells reminiscent of mammalian smooth muscle fibers. Muscle cells of frog atria were teased away from bundles incubated in EDTA Ringer's. An isolated, unfixed fiber is illustrated in Fig. 8. It can be seen to be a long tapering cell which is unbranched. All isolated cells were between 175 μ and 250 μ in length. These findings are in agreement with those on fibers isolated in KOH (Skramlik, 1921). Thus, it appears certain that the length of the atrial bundle isolated by the sucrose gap was greater than the length of a cell.

No structure completely analogous to the intercalated disc of mammalian cardiac muscle is present in frog atrial muscle (Figs. 10 and 13). In specimens fixed with MnO_4^- the individual muscle cells are seen to lie very close together. Desmosomes occur along adjacent cell membranes, particularly where the tapering ends of cells are closely packed. They occur less frequently along the lateral aspects of the cells. Myofibrils attach to the cell membrane by means of an expansion of dense material similar to that seen in mammalian intercalated

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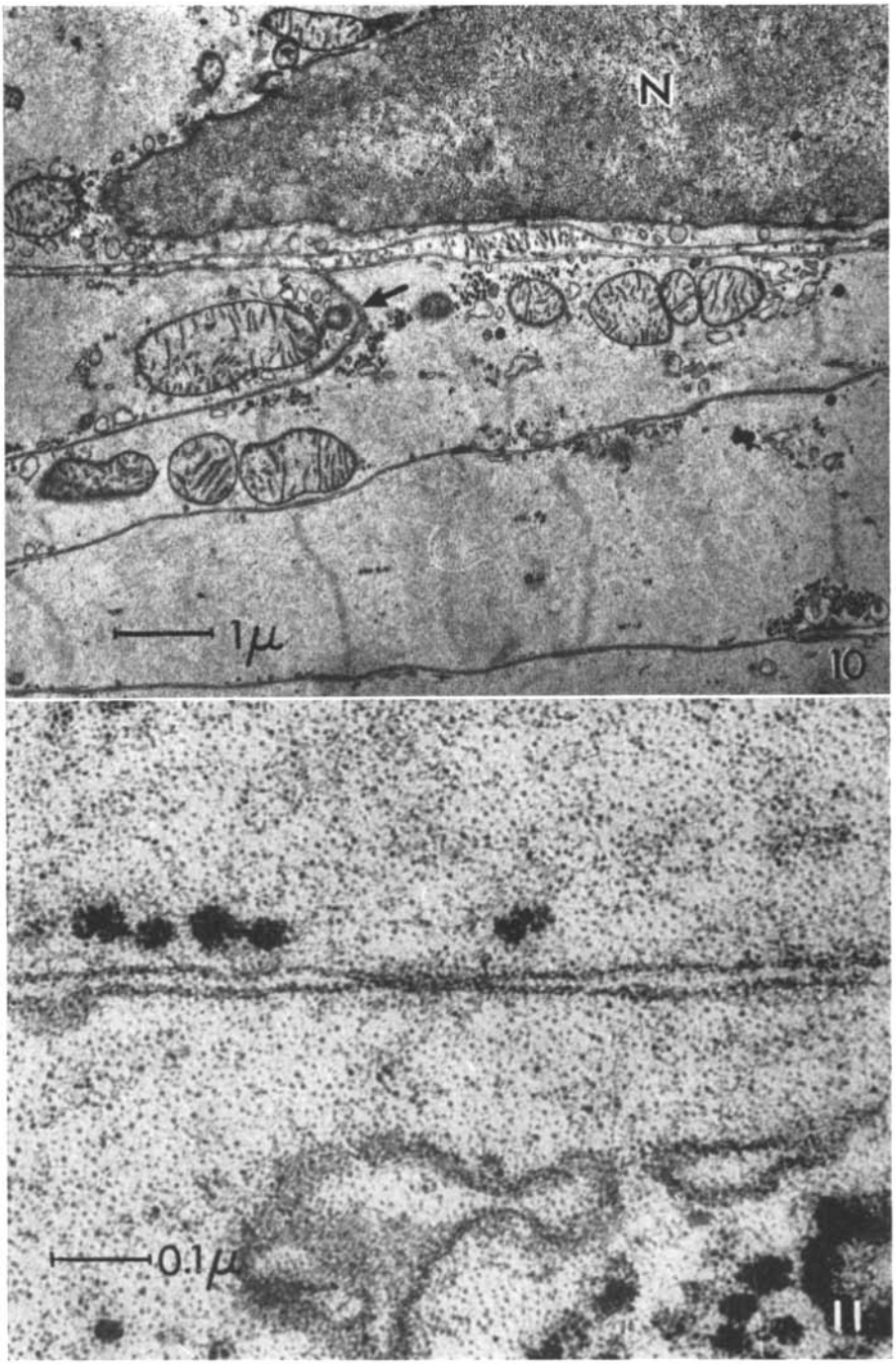
FIGURE 7. Frog atrial muscle. This photomicrograph shows an atrial trabeculum in longitudinal section. Individual muscle cells can be traced for a considerable distance in the bundle. Connective tissue elements are not readily identifiable between the cells. Such preparations usually show maximal contraction as evidenced by the pattern of cross-striations. Formol–sublimate-fixed, double-embedded, stained with Masson trichrome. × 2,000.

FIGURE 8. Frog atrial muscle. This is a bright field photomicrograph of a single, isolated atrial fiber or cell. The cells are long tapering spindles. No branching of fibers has been seen. The nucleus (arrow) occupies the central region of the cell. Cell isolated in Ringer's containing 0.02 per cent EDTA. × 500.

FIGURE 9. Frog atrial muscle. An atrial trabeculum in cross-section is illustrated in this photomicrograph. Each trabeculum contains something of the order of 100 muscle fibers or cells. The muscle cells are surrounded by a thin endocardium composed of endothelium (arrow) and a thin underlying layer of collagen fibrils. Each muscle cell has few myofibrils and it is thus difficult to differentiate individual cells with two or more myofibrils from the ends of cells in which only a single myofibril is present. Formol–sublimate-fixed, double-embedded, stained with Masson trichrome. × 2,000.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11

discs. This occurs both at the ends of cells and where the myofibrils make an oblique angle with the membrane. In addition, myofibrils are attached by similar dense material laterally where they are parallel to the membrane. These attachments are extensions of material from the Z band (Fig. 13).

The lack of basement membrane and the narrowness of the space between the cells throughout their extent make virtually the entire cell surface analogous to the intercalated disc of mammalian cardiac muscle. In MnO_4^--fixed material, nexuses are seen in small regions along the cell membranes of adjacent cells (Fig. 11). They often occur directly adjacent to desmosomes, as is seen in the germinative layer of frog skin (Dewey and Barr, 1964). Thus far, nexuses between frog atrial fibers have been observed only rarely in specimens fixed in OsO_4. In fact, large gaps occurred between the cells in such specimens and even the desmosomes appeared partially pulled apart. This is probably due to the shrinkage of the cells in these preparations. Similar findings have been described for a number of tissues (Dewey and Barr, 1964).

Further comment needs to be made on the existence of an intercalated disc in frog atrial muscle. While all the components characteristic of an intercalated disc of mammalian cardiac muscle are present along the cell membrane of frog atrial fibers, they are not restricted to a discrete area of membrane between adjacent cells such that the composite of structures is identifiable as an intercalated disc with the light microscope. Therefore, an intercalated disc in frog atrial fibers cannot be identified. This view is in contrast to that presented by Poche and Lindner (1955) and by Fawcett and Selby (1958) who have designated intercalated discs in frog and turtle atria. It would seem more reasonable, however, to retain the original meaning of the term as it was developed through the application of light microscopic methods and restrict its use to organized structures similar to those in the mammalian cardiac fibers.

Incubation and fixation in various hypertonic solutions resulted in the ap-

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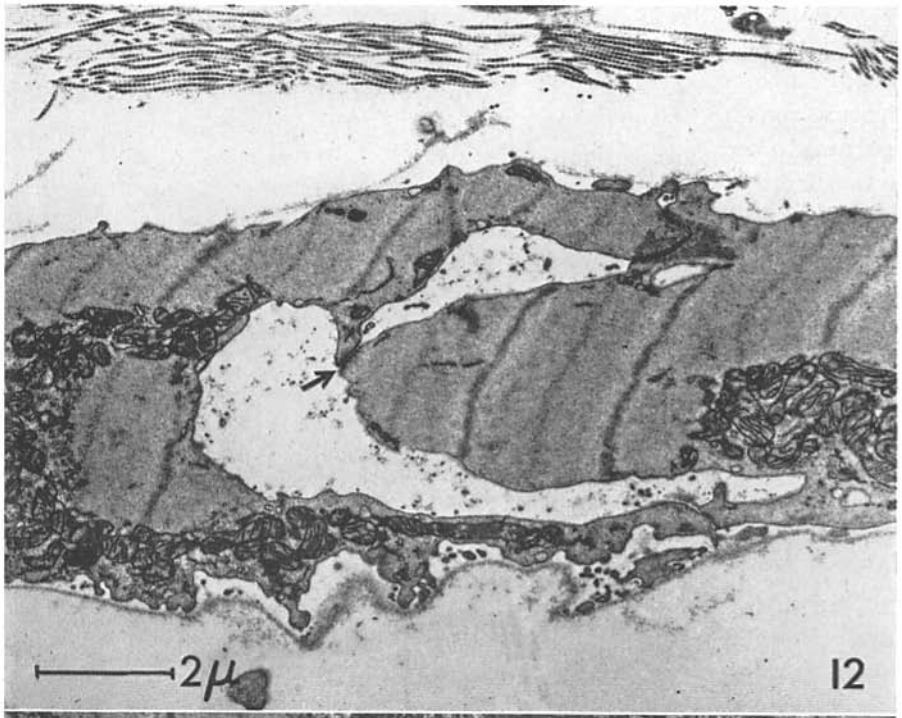
FIGURE 10. Frog atrial muscle. Portions of several atrial fibers are shown in this electron micrograph. The midregion of a cell with its nucleus (*N*) is shown. A narrow region of extracellular space containing collagen fibers separates this cell from those in the lower portion of the field. The lower group of cells is surrounded by a basement membrane and no connective tissue elements are seen between the cells. The blunted end of one cell is shown as it contacts another cell at the arrow. No intercalated discs are seen. While prolonged fixation with MnO_4^- disaggregates the myofibrils making them difficult to identify, the lower fiber can be seen to contain only one myofibril. This is often true near the long tapering ends of the cell. Incubated in isotonic sucrose solution, isosmotic MnO_4^--fixed. × 13,500.

FIGURE 11. Frog atrial muscle. This electron micrograph illustrates a small region of nexus between two atrial cells. Here the nexus occurs along the lateral surfaces of the cells and is not immediately adjacent to either desmosomes or regions of attachment of myofibrils to the cell membranes. Incubated in isotonic sucrose solution, isosmotic MnO_4^--fixed. × 130,000.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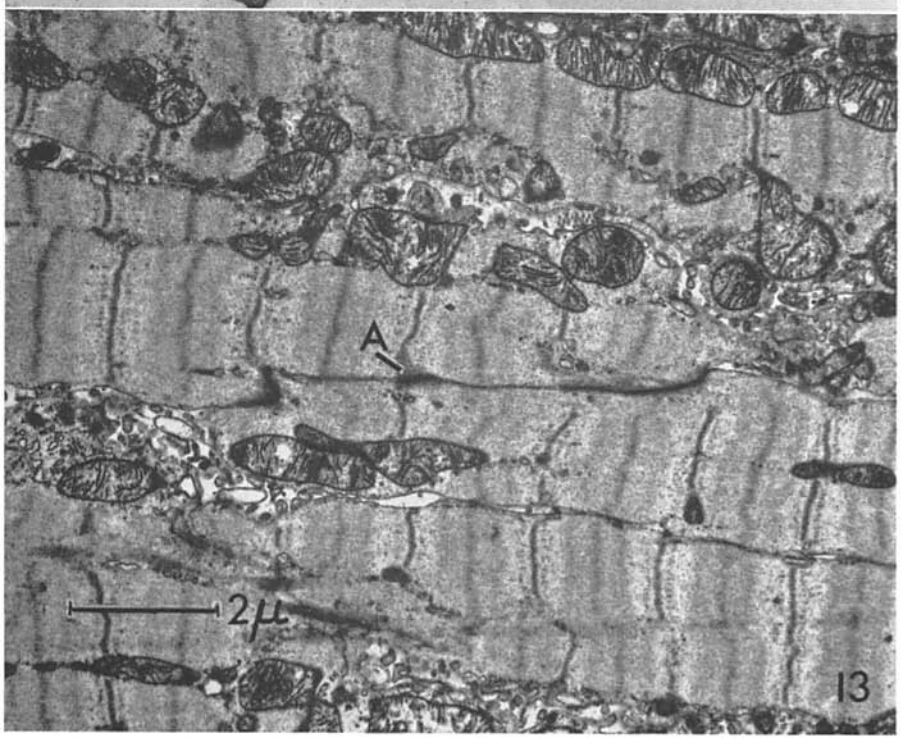
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pearance of large gaps between the cells except at desmosomes and regions of myofibrillar attachment (Fig. 12). All nexuses were ruptured. The desmosome seems to have considerable resistance to rupture. The effects of hypertonicity are completely reversible. In an attempt to determine whether the effect of hypertonic media on the cell contacts of frog atrial fibers was due to cell shrinkage or merely the effective water concentration, specimens were incubated prior to fixation in Ringer's solution made 630 mOsM by the addition of solid urea. As seen in Fig. 13, incubation in this hyperosmotic but isotonic solution had no effect on the intercellular relationships.

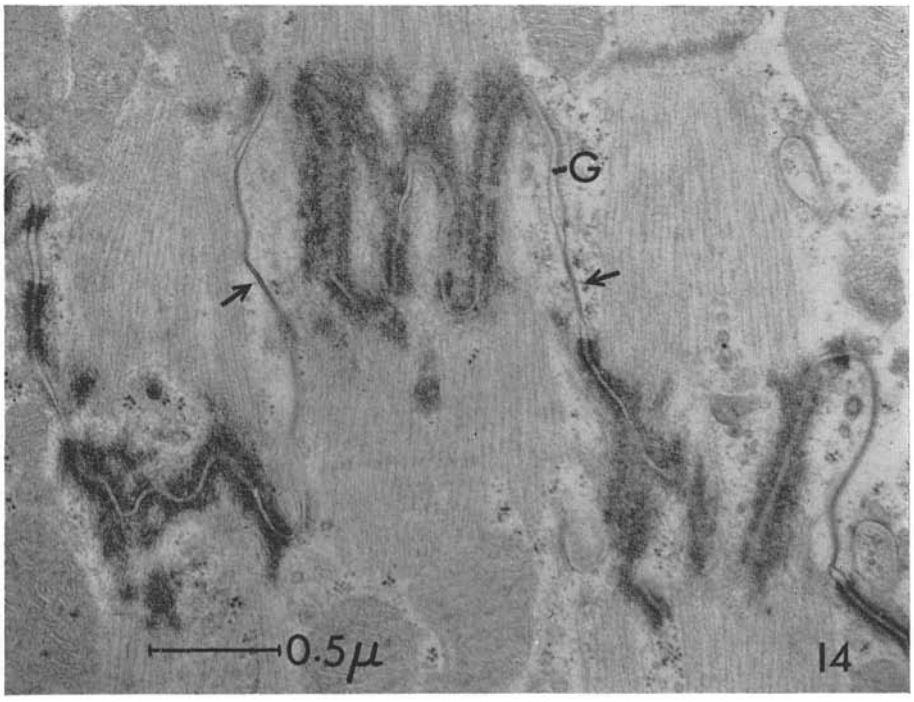
The Intercalated Disc of Mammalian Cardiac Muscle

Although frog atrial muscle is functionally similar to other vertebrate cardiac muscles, it is quite different morphologically from mammalian ventricular muscle. Thus, since the morphology of the mammalian intercalated disc is better known, the effects of hypertonic solutions on this structure were investigated in order to strengthen the conclusions drawn from data on frog atrium.

Fig. 14 illustrates the general ultrastructural features of the intercalated disc of guinea pig cardiac muscle. The disc can be subdivided into two major regions: (*a*) intersarcoplasmic and (*b*) interfibrillar. The interfibrillar portion of the disc is characterized by attachment of the myofibrils of adjacent cells to the cell membranes. These regions generally lie normal to the axis of the fiber. The intersarcoplasmic portion of the disc is characterized by regions of specialization along the adjacent cell membranes; *i.e.*, nexuses and desmosomes. The intersarcoplasmic portion of the disc may lie normal to the axis of the fiber or it may be parallel to the axis of the fiber for a distance of one or more sarcomeric levels. The result is that intercalated discs transect the fiber in a stepwise fashion. The adjacent cell membranes of the disc along most of the intersarcoplasmic region are separated by an intercellular gap of 100 to 200 A. This gap is often seen to be continuous with the extracellular space at the

FIGURE 12. Frog atrial muscle. This electron micrograph illustrates the effect of incubation in hypertonic sucrose solution followed by hypertonic fixation on amphibian cardiac fibers. Considerable shrinkage of cells occurs with a wide extracellular gap between the cells. All nexuses between the cells have been disrupted. As in mammalian cardiac fibers, these atrial fibers adhere only at desmosomes (arrow) and at regions of attachment of myofibrils. Hypertonic sucrose incubation, hyperosmotic MnO_4^--fixed. × 8,800.

FIGURE 13. Frog atrial muscle. The specimen illustrated in this electron micrograph was incubated in Ringer's solution made hyperosmotic with urea. Such specimens show no differences in intercellular relationships from those incubated in normal Ringer's solution. Urea does seem to retard fixation by MnO_4^- so that disaggregation of myofibrils is not as marked as in controls. Attachment of a myofibril laterally to the membrane by expansions of dense material from the Z band is seen at *A*. Incubated in hyperosmotic Ringer's solution containing urea, hyperosmotic MnO_4^--fixed. × 10,000.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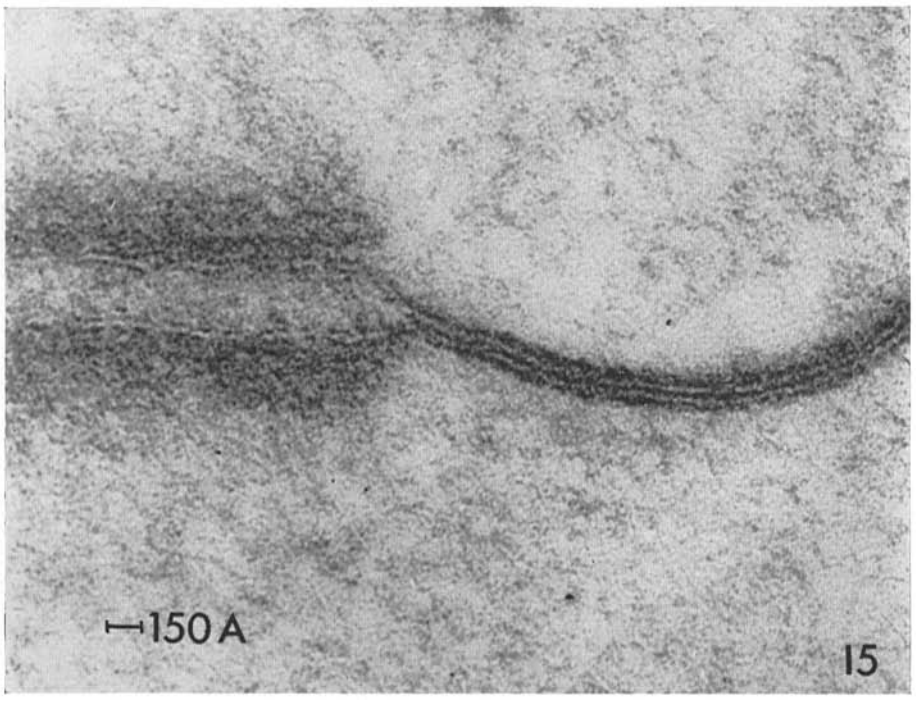
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margin of the disc. The desmosomes which occur along the intersarcoplasmic portion of the disc are more commonly found along the areas which lie normal to the axis of the fiber. The membranes in the region of the desmosome are usually separated by a distance greater than that separating the cell membranes elsewhere along the disc. In MnO_4^--fixed specimens (Fig. 15) dense material is seen to span the gap between the adjacent cell membranes at desmosomes instead of the fibers seen in OsO_4-fixed specimens. In other regions along the intersarcoplasmic portion of the disc the adjacent cell membranes are fused along their outer leaflets to form a nexus (Fig. 15). The nexuses along the intersarcoplasmic regions parallel to the fiber axis often run the length of one sarcomere. Nexuses normal to the fiber axis involve much smaller areas.

Incubation and fixation of cardiac fibers in hyperosmotic media disrupted the intercalated disc as illustrated in Fig. 16. Considerable shrinking of the fiber occurred as evidenced by the increased density of the sarcoplasm and general compactness of sarcoplasmic organelles such as mitochondria and sarcoplasmic tubules between myofibrils. The intercellular gap between adjacent cell membranes along the disc was widened. Separation of the membranes of the nexal regions occurred so that the gap appeared usually to run all the way along the disc. More shrinkage was encountered in the periphery of the specimen than towards its center. Thus, fibers were observed in which occasional nexuses were still present along the disc. Adherence of adjacent membranes at desmosomes was maintained. The membranes at desmosomes were more than normally separated after hypertonic treatment but the dense material between the membranes was still apparent. Therefore, it would appear that desmosomes are sites of mechanical adherence between cardiac muscle cells. The separation of nexal membranes due to hypertonicity was completely reversible. Specimens which were soaked in hypertonic media and then returned to normal K-H solution prior to fixation with a 320 mOsM

FIGURE 14. Guinea pig papillary muscle. A small portion of an intercalated disc is illustrated in this electron micrograph. Myofibrils attach to the membranes of adjacent cells at the interfibrillar region of the disc as prominent, dense, finely fibrous masses. The space between cell membranes at these regions is from 100 to 200 A. In the intersarcoplasmic regions of the disc, which here lie parallel to the fiber axis, nexuses (arrows) and desmosomes occur along the adjacent membranes. In addition, a gap between the membranes greater than 100 A is always present in the non-nexal regions of this portion of the disc (*G*). Incubated in isotonic K-H solution, isosmotic OsO_4-fixed. × 33,000.

FIGURE 15. Guinea pig papillary muscle. This electron micrograph illustrates an even smaller region of the intersarcoplasmic portion of an intercalated disc. A desmosome is shown in the left-hand portion of the figure. The cell membranes immediately adjacent to the desmosome fuse to form a nexus. Incubated in isotonic K-H solution, isosmotic MnO_4^--fixed. × 320,000.

fixative were indistinguishable from those which had been soaked in normal K-H prior to fixation.

The effects of hypotonic solutions on the structure of the intercalated disc are more difficult to assess. From the observations made so far, it would appear that swelling increases the nexal area. However, until an accurate

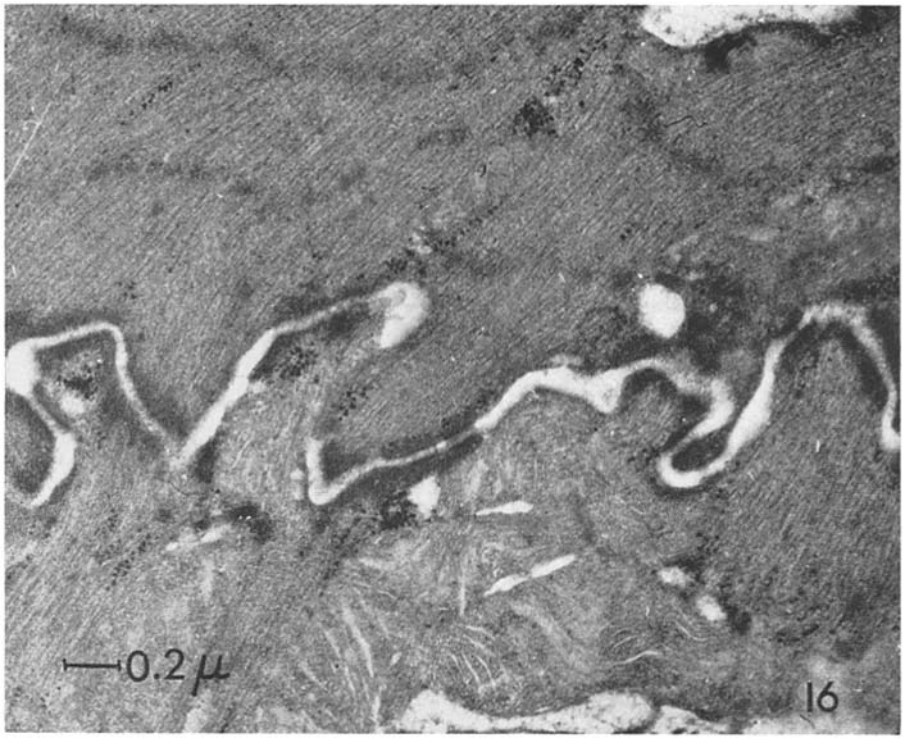


FIGURE 16. Guinea pig papillary muscle. The effect of incubation in hypertonic sucrose solution on the intercalated disc is illustrated in this electron micrograph. The gap between adjacent cell membranes along the disc widens with complete rupture of nexuses. Fine fibrous material spans between the membranes at both myofibril attachment sites and desmosomes. Incubated in hypertonic K-H solution, hyperosmotic OsO_4-fixed. × 29,000.

method for quantifying the surface area of the disc involved in nexuses is available for analyzing such preparations, the extent of the increase cannot be known.

Ferrocyanide Space in Guinea Pig and Frog Cardiac Muscle

The relative amount of extracellular space is difficult to assess from electron micrographs. As yet no quantitative method has been applied to determine the amount of shrinkage or swelling that occurs in specimens during

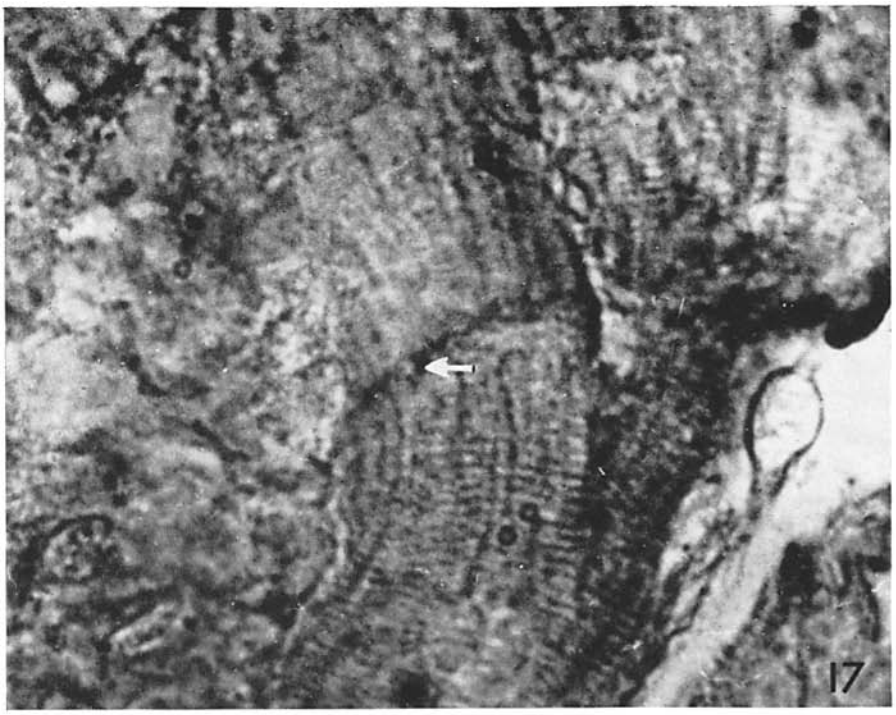
fixation, dehydration, and embedding. This is particularly pertinent when considering the surface area between cells involved in a nexus. It is of interest to know whether the *in vivo* nexal area in mammalian cardiac muscle along the intercalated disc is more extensive than observed here or whether it forms a seal around the adjoining cells separating the central portion of the disc from the rest of the extracellular compartment. As can be seen from Fig. 17, ferrocyanide readily penetrated into the intercalated disc and set it in sharp relief. Thus, it would appear that the space observed between the adjacent cell membranes is in continuity with the extracellular space. Further, it is interesting to note that ferrocyanide penetrated the tubular system within the cell somewhat as ferritin penetrates the system in frog skeletal muscle (Huxley, 1964).

In view of the relatively small surface area involved in nexuses between frog atrial fibers, it was essential to obtain some idea whether the cells were being separated by the rigors of fixation and subsequent treatment. Fig. 18 illustrates the fact that sufficient extracellular space exists between frog atrial fibers to allow the penetration of ferrocyanide so that individual cells are delineated. Such observations are particularly convincing since frog atria incubated in ferrocyanide containing Ringer's solution continue to beat at a normal rate throughout the duration of the experiment.

DISCUSSION

Propagation of action potentials across inactive portions of atrial bundles in sucrose demonstrates that transmission between amphibian atrial cells is electrical. In other words, the interiors of these cells are connected by pathways of low enough resistance to allow electrical transmission of action potentials with a large safety factor. This is demonstrated by experiments in which action potentials jump across a sucrose gap of as much as 600 μ in spite of the fact that the excitatory current density is much less than it is *in vivo*. The excitatory current density is low not only because of the resistance of the segment in the gap but also because of the increased myoplasmic resistance of the segments exposed to sucrose due to the appreciable loss of intracellular ions. The segment in the gap shows no electrical or mechanical response. In fact, if soaked long enough, it loses the ability to respond even when returned to Ringer's solution. The "all-or-none" nature of the propagation past the gap as the shunt resistance is decreased makes it difficult to imagine an intermediary "chemical transmission" step as has been advocated (Sperelakis, Hoshiko, and Berne, 1960). On the contrary, the segment of muscle in the sucrose gap appears to behave simply as a core conductor connecting the segments in the Ringer's solution pools.

The fact that the longitudinal resistance increases drastically during the same treatment that causes the nexal membranes to separate argues strongly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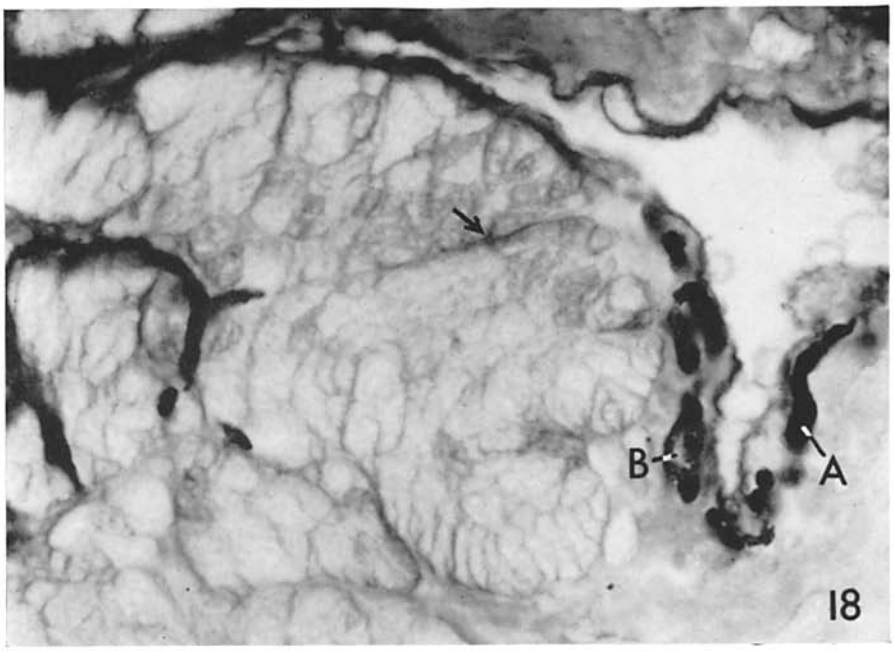
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that the nexuses are the low resistance connections between the cells. This conclusion is strengthened by the observation that both the structural and functional alteration in hypertonic solutions is reversed upon returning to normal solutions. Moreover, when the tissues were exposed to Ringer's solution to which enough urea had been added to increase the osmolarity three times, there was no effect on the structure of the nexuses and propagation of action potentials continued indefinitely. The argument could be made that there are pockets of extracellular fluid sealed between cells by continuous nexuses, particularly at the intercalated disc. However, there is no anatomical evidence for this. In fact, in the guinea pig cardiac muscle a nexal region is not always found in the regions of the disc closest to the interfiber space. Moreover, in the amphibian atrial bundles there are no intercalated discs as such at all, only spotlike nexal and desmosomal regions. These occur around all aspects of the fusiform cells. In this tissue especially it seems unlikely that there are any isolated pockets of fluid between cells. Finally and conclusively, ferrocyanide ion diffuses quickly along the intercalated discs and between frog atrial fibers. Conversely, it seems that the ions of the non-nexal region should be washed away by the flowing sucrose solution. Of course, the endothelial layer tends to slow this, but wherever ferrocyanide diffuses, sucrose should be able to do so also and the extracellular ions of these regions should diffuse away. These considerations also oppose the notion of chemical transmission since, as Eccles and Jaeger (1958) point out, the post-junctional membrane potential can influence the rest of a cell only by way of a low resistance extracellular pathway. Thus, for fibers surrounded by sucrose, electrotonic spread from any hypothetical junctional receptor site to the rest of the cell is not possible.

FIGURE 17. A longitudinal section of guinea pig papillary muscle incubated in a K-H solution containing ferrocyanide. The degree of penetration of ferrocyanide between and into mammalian cardiac fibers is illustrated here. Most of the ferrocyanide has been washed out of the larger extracellular spaces prior to fixation. The ferrocyanide is precipitated, converted to Prussian blue, and appears dark in this photomicrograph. It clearly delineates the intercalated disc in the center field (arrow) which indicates that the space along the disc is continuous with the space between fibers. In addition, it penetrates the sarcoplasmic tubular system of this muscle. Thus, the fiber in the center of the field appears cross-striated. × 2,500.

FIGURE 18. A cross-section of frog atrial wall incubated in Ringer's solution containing ferrocyanide. Most of the ferrocyanide has been washed out of the larger extracellular spaces prior to fixation. This photomicrograph illustrates the extent to which ferrocyanide penetrates between frog atrial fibers. The heavier dark lines (arrow) indicate the extracellular spaces in the connective tissue septa between muscle bundles. Individual muscle cells are delineated by fine lines of Prussian blue. The larger dark masses such as those at *A* and *B* are melanophores. × 1,250.

Lability of the Nexus

Previously reported data (Dewey and Barr, 1962) indicate that the nexus is a relatively unstable structure. Sedar and Forte (1964) have shown that Ca^{++} removal by EDTA can cause separation of the membranes of nexuses (tight junctions) and desmosomes between intestinal epithelial cells. The nexuses between dog intestinal smooth muscles were easily demonstrable only after an incubation similar to that necessary for physiological and pharmacological responsiveness. The nexuses of several smooth muscles seem to be more stable. The nexuses of guinea pig cardiac muscle are probably the most stable of all those examined but they quite obviously are labile in hypertonic solutions.

It is conceivable that the structure of the nexus is affected by the osmolarity of the bathing solution. On the other hand, from the urea experiments it would seem that the tonicity of the solution is the important factor, not the chemical activity of the solvent water. This could be used to argue that the nexal membranes were simply pulled apart by the tension developed during cell shrinkage.

The same mechanism of propagation of action potentials apparently obtains for vertebrate visceral smooth muscle although the data are not quite so impressive. Nagai and Prosser (1963) have reported that the resistance between the interiors of neighboring smooth muscle cells increases when the osmolarity of the bathing physiological saline is increased by addition of sucrose. The nexuses between the smooth muscle cells of the taenia coli of guinea pig and the circular layer of feline intestinal jejunum are ruptured by incubation in K-H solutions made 912 mOsM by addition of solid sucrose (Dewey, 1964).

General Implications

The exact cell-to-cell relationships in electrogenic tissues have been the objects of controversy for both anatomists and physiologists for a century. The issue of the discrete nature of the vertebrate neuron was largely settled in the first quarter of the 20th century (Ramon y Cajal, translation, 1954). On the other hand it has been only recently that cardiac (Sjöstrand and Andersson, 1954) and smooth (Bergman, 1958) muscle have been shown to be composed of separate cells. The dimensions of the extracellular fluid layer in the intercellular gap between cells, if any, are important for the understanding of cell-to-cell transmission. Since DuBois Reymond, physiologists have been drawing hypothetical patterns of current flow between excitable cells. Consideration of any gap of highly conducting extracellular fluid obviously should have high priority in such model making. For example, the space between the squid giant axon and its Schwann cell is very small (100 A) (Villegas *et al.*, 1963); so

is the synaptic cleft of the giant synapse of the squid (Hagiwara and Tasaki, 1958), but in both cases there is no electrical coupling between the cells. In fact, from the requirements of chemical transmission, Eccles and Jaeger (1958) have discussed in considerable detail the necessity for a gap to provide a current pathway between the chemically activated postsynaptic membrane and adjacent electrically excitable membrane areas. Thus, the occurrence of an intercellular gap favors chemical transmission and makes electrical transmission unlikely. On the other hand, the occurrence of a nexus would favor electrical transmission and make chemical transmission unlikely.

In this regard, Woodbury and Crill (1961) have approached the problem of action potential propagation in the rat atrium by assuming that the gap between cardiac cell membranes at an intercalated disc is everywhere 80 A. The ratio of the area of apposition to gap distances in this case is so large that one might expect transmission to occur easily. However, their analysis showed that under these conditions propagation of action potentials required the disc membrane to have an extremely low resistance (*i.e.* 1/1000 of the plasma membrane). However, since there is complete exclusion of the extracellular fluid at the nexal region of the disc, one would predict that the nexal membrane could have a resistance much closer to that of the plasma membrane. This would be more in keeping with space constant measurements.

The issue is even clearer at the nexus between smooth muscle cells where the cross-sectional area is much less (Dewey and Barr, 1962; Dewey and Barr, 1964; Oosaki and Saburo, 1964). In this situation no reasonable membrane resistance would allow electrical transmission to occur across an intercellular gap and it would seem necessary to postulate either protoplasmic continuity or the nexus to explain the electrophysiological data. (Barr, 1961, 1963).

The septum in earthworm median giant axon is in a sense the simplest kind of synapse. Because Sherrington defined the synapse (Foster, 1897; Fulton, 1949) in terms of the morphology of the central nervous system of vertebrates, the term has been associated with unidirectional transmission of excitatory and inhibitory influences. Thus, heretofore, there has been reluctance to accept the septa between the segmental nerve cells of crayfish and earthworms as synapses because of their two-way transmission (Eccles, 1964; McLennan, 1963). However, the septate junctions in the crayfish nerve cord and, in particular, the lateral giant axon to giant motor axon of the third root of abdominal ganglia are ordinarily referred to as synapses. In fact, the lateral-to-motor is now perhaps the classic example of an electrically transmiting synapse (Furshpan and Potter, 1959). The nexus occurs at the median-to-motor synapse (Robertson, 1961), as well as at other electrical synapses (*e.g.*, the lateral giant axon to lateral giant axon and the lateral giant axon septa) in the crayfish giant motor system. Using OsO_4 as a fixative, both Robertson (1961) and Hama (1959, 1961) have commented on the narrowness of light

space between the dark lines in their electron micrographs of the crayfish and earthworm giant fibers. Although Hama's studies on known electrical synapses do not provide direct evidence for fusion of pre- and postsynaptic membranes, the over-all thickness of these OsO_4-fixed synapses is comparable to that of the MnO_4^--fixed nexuses in vertebrate muscles, epithelia, and the earthworm axon (Dewey and Barr, 1964). Thus, it would seem likely that electrical coupling between cells, in general, is to be correlated with the nexus. Moreover, there is a spectrum of functions at electrical synapses, including: one-way transmission (Furshpan, 1964; Furshpan and Potter, 1959; Martin and Pilar, 1963 *a*, *b*), two-way transmission (Bennett *et al.*, 1963; Kao and Grundfest, 1957; Martin and Pilar, 1963 *a*, *b*; Watanabe and Grundfest, 1961), facilitation (Watanabe and Bullock, 1960), and inhibition (Furukawa and Furshpan, 1963). In this light, it would appear reasonable to include the simple septal junctions as synapses since they probably all have the same nexal structure. This usage would conform to Sherrington's original definition of a synapse as a junction between nerve cells. Synapses might then be found to divide easily into electrical (nexal) and chemical (separated) synapses.

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