Correlations between Electron Microscopic and Physiological Observations in Heart Muscle

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The contraction of vertebrate heart muscle differs in characteristic ways from that of skeletal muscle (Brady, 1967): cardiac muscle cannot be tetanized; it has a significant resting tension at lengths where normal contractile tension is developed, a resting tension which may be subject to certain self-regulatory control mechanisms; and it is characterized by a slow onset of contractility. Physiologists have recently restudied these differences by more quantitative methods (Edman, Grieve, and Nilsson, 1966; Brady, 1966; Sonnenblick, 1967).

The renewed interest in a quantitative description of cardiac contraction coincides with notable advances in the understanding of one aspect of the contractile process in skeletal muscle: the link between the excitatory electrical event at the cell surface (the action potential) and the mechanical events of contraction and relaxation in the interior of the cell. Investigation of frog skeletal muscles has led to significant correlations between cellular membrane systems as revealed by electron microscopy (Porter and Palade, 1957; Peachey, 1965), by electrophysiologic experiments on cell surface and intracellular membranes (Huxley and Taylor, 1958; Costantin and Podolsky, 1967), and by biochemical reactions of fragmented intracellular membranes (Hasselbach, 1964). These correlations form the basis of a model for the mechanisms by which excitation is coupled to contraction in amphibian skeletal muscle.

It has been widely assumed that the model for excitation-contraction coupling, based on experiments on frog skeletal muscle, can be applied with relatively minor modifications to mammalian heart muscle. The present paper will review ultrastructural evidence from our own and other laboratories suggesting that this assumption is incorrect. The paper will describe additional experiments designed to identify the artifacts by which fixation for electron microscopy distorts the tissue compartments concerned with excitation-contraction coupling in heart muscle; and experiments designed to define these compartments under physiological conditions. These additional experiments contribute to a critique of the methods on which correlations between electron microscopic and physiologic data depend.

THE SKELETAL MUSCLE PATTERN OF EXCITATION-CONTRACTION COUPLING

The classical pattern (observed in frog's skeletal muscle of the twitch type) for the coupling between the action potential at the cell surface and the mechanical events (contraction and relaxation of the myofibrils) in the interior of the muscle cell may be divided for convenience into at least four steps, as shown in Fig. 1. (1) The action potential sweeps across the longitudinal cell surface or sarcolemma. (2) The message that an action potential is passing across the cell surface is transmitted toward the interior of the cell at right angles to the cell surface. The transmission, presumably

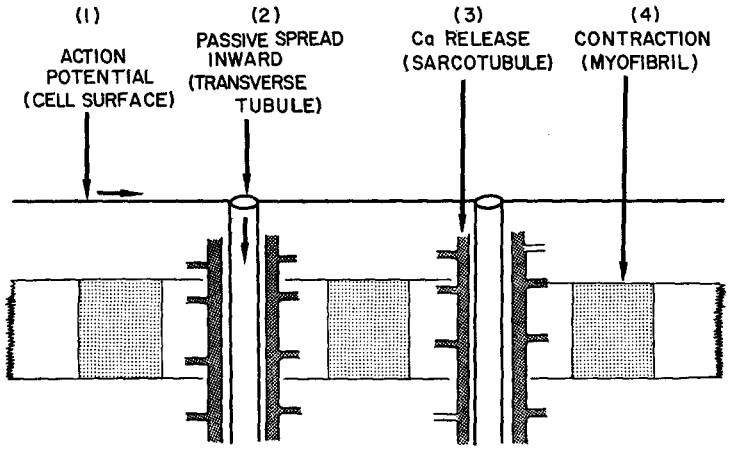

FIGURE 1. A schematic representation of four steps in the excitation-contraction coupling sequence of frog (twitch type) skeletal muscle. See text for explanation.

by cable spread, occurs via the transverse tubules, that is, via the roughly cylindrical infoldings of the plasma membrane lining the cell surface, infoldings which are, in effect, extensions of the extracellular compartment. (3) The electrical signal transmitted down the transverse tubule is translated by calcium-storage depots lying near the transverse tubule as a command to release stored calcium into the cytoplasm. These calcium-storage depots are specialized parts of a second tubular network which has been variously designated in published studies as sarcotubules, longitudinal tubular system, and sarcoplasmic reticulum. (4) The increase in the cytoplasmic calcium concentration in the immediate environment of the myofibrils triggers myofibrillar contraction. The four steps are then reversed in response to an as yet unidentified signal and by an as yet unidentified sequence.

The ultrastructures which act as cellular depots for the storage of calcium play a central role in this scheme. In frog skeletal muscle of the twitch type, the organelles which, on the basis of the available experimental evidence, are presumed to be calcium-storage depots (Costantin, Franzini-Armstrong, and Podolsky, 1965) are confined to positions along the transverse tubules. In particular, these organelles are not found at the cell surface where the action potential occurs. The absence of sub-

sarcolemmal depots suggests that in frog skeletal muscle the action potential cannot directly release calcium ions into the cytoplasm. Instead, an electrical event in the transverse tubule must precede the release of calcium from the calcium-storage depots.

ULTRASTRUCTURES IMPLICATED IN EXCITATION-CONTRACTION COUPLING IN MAMMALIAN HEART MUSCLE

The ultrastructural systems for excitation-contraction coupling in mammalian heart muscle and frog (twitch type) skeletal muscle may be compared on the basis of two types of observations: (*a*) studies of the distribution and geometrical arrangement of the transverse tubules and calcium-storage depots with respect to one another and to the plasma membrane at the longitudinal and transverse cell surfaces, and (*b*) measurements of the dimensions of the transverse tubules. The distribution of calcium-storage depots can be evaluated solely on the basis of electron micrographs. Estimates of the dimensions of transverse tubules must, in addition, take into account the distortion of the physiologic dimensions which is introduced during the procedures by which heart muscle is prepared for electron microscopy.

Distribution of Calcium-Storage Sites in Mammalian Heart Muscle

The sarcotubules of mammalian heart muscle contain structurally specialized regions which correspond morphologically to the structures identified as calcium-storage depots in frog skeletal muscle (Fig. 2). As in frog skeletal muscle, these presumptive sites of calcium storage are located along the cytoplasmic surfaces of the plasma membrane lining the transverse tubules (Simpson and Oertelis, 1962; Nelson and Benson, 1963; Simpson, 1965; Page, 1967). However, the distribution of these sites in mammalian heart muscle differs from the frog skeletal muscle pattern in a critical respect: in mammalian heart muscle, morphologically typical specializations of the sarcotubules are arranged not only along the cytoplasmic surfaces of the transverse tubules, but also along the cytoplasmic surfaces of the plasma membranes lining the longitudinal surfaces of the cell (Page, 1966, 1967). This observation means that in this tissue at least some of the presumptive calcium-storage depots lie in proximity to the site of the action potential. Heart muscle therefore differs from skeletal muscle in that the cardiac action potential may at least in theory activate the release of calcium ions into the cytoplasm directly without an intervening electrical signal involving the transverse tubule.

On the basis of the distribution of calcium-storage sites in Purkinje cells from the cat's papillary muscle, it was predicted that excitation-contraction coupling in mammalian heart muscle cells must differ in critical respects from that in frog skeletal muscle cells (Page, 1966, 1967). This prediction has received suggestive independent confirmation in electrophysiological experiments by Müller (1966). Müller attempted to repeat in heart muscle the classical experiments of Huxley and Taylor (1958) in frog skeletal muscle. Huxley and Taylor had applied sharply localized depolarizing currents of subthreshold intensity to the cell surfaces of various skeletal muscles. They obtained local contractions of the myofibrils which lie beneath the sarcolemma

when they depolarized certain sensitive areas of the cell surface, but not when they depolarized other areas. Huxley and Taylor identified the depolarization-sensitive spots with the openings at the cell surface of structures which present-day terminology refers to as the transverse tubules. Sharply localized openings of cardiac transverse tubules can also be identified in mammalian heart muscle (Raynes, Simpson and Bertaud 1967). Nevertheless, the technique of Huxley and Taylor does not produce local activation of cardiac myofibrils. Instead, Müller (1966), working with preparations of trabecular muscle from the hearts of dogs and sheep, was able to elicit only a widespread contraction of all the sarcomeres in the microscopic field under observation.

One interpretation of Müller's result is that subthreshold depolarizations in mammalian heart muscle activate calcium release from calcium-storage depots immediately

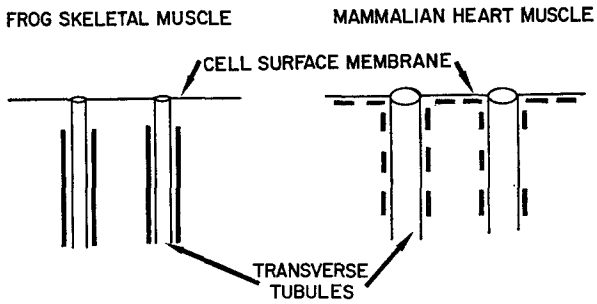

FIGURE 2. Comparison of the distribution of depots specialized for storage of calcium in frog twitch-type skeletal muscle (left) and mammalian heart muscle (right). Calcium-storage depots are shown as black bars.

below the sarcolemma. These storage depots can be shown in serial-section electron micrographs to be part of a single sarcotubular network (Page, 1967) which is continuous across the Z bands separating one sarcomere from its neighbors (Simpson, 1965). Recent electrophysiological experiments on "skinned" frog skeletal muscle cells (Costantin and Podolsky, 1967) indicate that the sarcotubular system can respond to stimulation both by graded and by "all-or-none" responses. It is conceivable that by stimulating the cell surface of heart muscle preparations, Müller may have elicited responses of this type.

In mammalian heart muscle the organelles here referred to as calcium-storage depots are always found in close association with the cytoplasmic surface of a plasma membrane. In this context, a plasma membrane is one which satisfies the electron microscopic criteria for a plasma membrane, as described by Sjöstrand (1963). Such plasma membranes and the associated calcium-storage depots are observable at the cell surface, along the transverse tubules, along longitudinal extensions of the transverse tubules, and at the intercalated discs. At the intercalated discs, which constitute the transverse cell boundaries of cardiac cells, the plasma membranes of two adjacent cells may fuse to form a tight junction. In heart muscle, as in other tissues, tight junctions are known to be surfaces which present a low resistance to the passage of electric current (Barr, Dewey, and Berger, 1965). It is therefore of interest that

calcium-storage depots are not found in association with the tight junctional portions of the intercalated disc, but are confined to those parts of the disc at which the plasma membranes of adjacent cells have not fused (Fig. 3). The calcium-storage sites of the sarcotubular system therefore appear to be associated with plasma membranes having a high electrical resistance, and are absent at plasma membranes having a low resistance. These observations are consistent with the idea that a depolarization of the overlying plasma membrane is normally required to act as a trigger for the release of calcium from such depots.

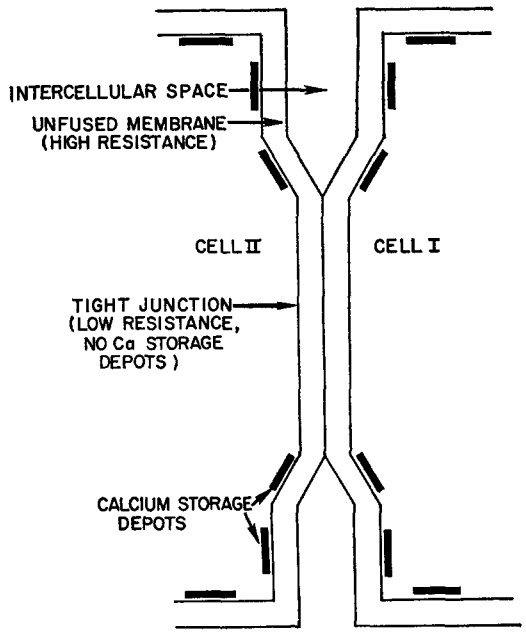

FIGURE 3. A schematic representation of the distribution of calcium-storage depots (shown as black bars) at a transverse boundary (intercalated disc) between two adjacent cardiac cells. Plasma membranes in the region where fusion has not occurred are shown as three-layered structures consisting of a light layer sandwiched between two parallel dark layers. At the tight junction the outer (noncytoplasmic) leaves fuse. Calcium-storage depots have not been found in the cytoplasm adjacent to the fused membrane.

The Transverse Tubular System

One of the questions raised by the application to heart muscle of the frog skeletal muscle model for excitation-contraction coupling is the role of the transverse tubules. In mammalian heart muscle transverse tubules recur periodically at the level of the Z band of the underlying myofibril (Simpson and Oertelis, 1962). It is generally agreed that the diameters of transverse tubules in mammalian heart muscle greatly exceed the diameters of the corresponding structures in frog skeletal muscle. Nevertheless, a reliable estimate of transverse tubular dimensions is not available for any mammalian cardiac cell. Such an estimate is, however, necessary for the critical evaluation of the skeletal muscle model in heart muscle. The model postulates that an action potential at the cell surface makes its presence known to the calcium-storage depots (located along the cytoplasmic surfaces of the transverse tubular membranes) by cable conduction down the transverse tubules. To test the model, it is necessary to determine whether passive spread by cable conduction can transmit the required "message" fast enough and far enough to be consistent with the known

electromechanical latencies of cardiac cells. A test of the model thus requires the application of the classical cable analysis of Hodgkin and Rushton (1946). This analysis, in turn, necessitates the assumption of a figure for the internal diameter of the transverse tubules, the other parameters of the cable equations being taken from values in the literature for cardiac muscle (Weidmann, 1952; Fozzard, 1966).

It is desirable to know the dimensions of the transverse tubules because of physiological considerations other than those imposed by the requirements of a cable analysis: (*a*) It is not known whether the openings of the transverse tubules significantly restrict the interdiffusion of solutes and water between the muscle interspaces and the tubular lumina. If such restrictions do exist, the tubular orifice and the plasma membrane lining the tubule can be regarded as two membranes of different permeability arranged in series. Assuming net ion transport across the plasma membranes lining the tubular lumina, such a system can potentially give rise to net flows of solution, solute, or solvent (Curran and MacIntosh, 1962; Patlak, Goldstein, and Hoffman, 1963). (*b*) It is possible that net movements of ions into and out of the lumina across the tubular membranes take place during excitation. Since the tubular lumina are unstirred, such net movements may give rise to transient concentration gradients. The significance of such gradients, as well as the time constants for their development and relaxation, would depend on tubular dimensions. (*c*) As has recently been pointed out by Teorell (1965) in a different context, the surface conductance in a cylindrical tube lined by fixed-charge membranes depends on the diameter of the tube. (*d*) It seems worth while to consider the possibility that in the course of cardiac cellular contractions significant hydrostatic pressure gradients may develop between the lumina of the transverse tubules and the tissue interstices, between the lumina of the transverse tubules and the cytoplasmic solution, or between the lumina of the transverse tubules and the lumina of the calcium-storage depots. (*e*) One may question whether the dimensions of the transverse tubules are constant during the cyclic contraction and relaxation of cardiac cells. It is conceivable that solution may be transiently expressed from the tubular lumina during one part of the cardiac cycle. Tubular dimensional changes of this type, although not accessible to experimental approaches so far developed, are potentially important. They would entail movements of the luminal electrolyte solution with reference to the electrically charged membrane lining the tubules. In this way they could give rise to various electrokinetic phenomena whose physiologic consequences have been extensively discussed by Teorell (1959, 1966).

Because of the high resolution of electron microscopy, visualization of cardiac transverse tubules by this method is easier than by other methods. However, the dimensions of the tubules in electron micrographs may differ from the dimensions prevailing in living hearts because of distortions introduced during fixation, dehydration, ultramicrotomy, and exposure to the electron beam. To evaluate these distortions, we have studied the effects of these preparative procedures on tissue compartments and composition of heart muscle and have used the results of this study to develop more effective methods for fixing heart muscle.

Tissue Compartments in Unfixed Mammalian Heart Muscle

To obtain a basis of comparison for the effects of electron microscopic fixation, we undertook a more complete, in vitro, compartmental analysis of unfixed heart muscle (Page and Gross, 1967; Page and Page, 1968.) For this purpose, left ventricles from rat hearts were perfused through the coronary blood vessels with solutions containing various radioactive molecular probes. Some of the molecular species tested were commonly used extracellular tracers; others were molecules known to penetrate into cells. The results of this study were unexpected and surprisingly complex. It was found that all tissue water equilibrates rapidly with certain solutes commonly considered to enter cells (urea, glycerol). About 40% of tissue water equilibrates quickly with the extracellular tracers SO_4 and sucrose. An additional 20% equilibrates slowly with these two molecular species. The latter fraction of cardiac water content has an ionic composition which differs from that of an extracellular solution and resembles that of a cellular solution. Finally, about 40% of tissue water is apparently not accessible to sulfate and sucrose. Both the compartment which does not equilibrate with sucrose and sulfate and the compartment which is slowly penetrated by these molecules shrink in solutions made hypertonic with NaCl. These findings are not compatible with the conventional two-compartment model of heart muscle. They therefore complicate the interpretation of the effects of fixatives on cardiac membrane systems.

Nevertheless, it proved possible to obtain useful information about the effects of fixatives on cardiac membranes (Krames and Page, 1967, 1968). The experimental technique was to perfuse hearts for 10 min with a physiologic salt solution containing the fixative, then to postperfuse them for 30 min with physiologic saline containing an extracellular tracer. The left ventricles were then analyzed for their contents of K, Na, Cl, Ca, Mg, water, and extracellular tracer. The redistribution of ions and water in membranes pretreated with fixative and then exposed to physiologic solutions could thus be determined.

The usual technique for fixing heart muscle is to immerse the tissue in the fixative. This technique gives nonuniform fixation. Both the osmotic and the chemical effects of the fixative are greatest near the surface of the tissue, a continuous gradient of fixative concentration being established from the surface inward to the core of the tissue. (It is not usually practicable to wait for a diffusion equilibrium with respect to the concentration of fixative in the tissue, since the long times required to attain equilibrium are associated with a leaching out of cellular constituents at the surface, where the fixative concentration is initially highest.) By contrast, the perfusion technique used by us allows a uniform fixation and a uniform washing out of the fixative.

The results of the experiments show that the volumes of tissue compartments in fixed heart-muscle cells depend critically on the osmolality of the fixative and on other characteristics of the fixation procedure. The fixatives tested were formaldehyde and osmium tetroxide. It could be shown that neither of these compounds renders the plasma membrane indiscriminately permeable to cations and anions,

except at fixative concentrations higher than those which give optimal tissue preservation. Since semipermeability is at least partially preserved, net movements of cell water across the membrane can take place when the muscle is exposed to an anisosmolal fixative. In addition, even small concentrations of formaldehyde and osmium tetroxide bring about an exchange of cellular K for extracellular Na. These changes resemble those which cardiac cells undergo when active transport of Na is inhibited by cardiac glycosides, by lowered external K concentrations, or by low temperature (Page, Goerke, and Storm, 1964). Since such changes are usually associated with changes in cell volume, it seems probable that fixation distorts cell volume even if the fixative is isosmolal.

An interesting, incidental finding of this study is that cardiac muscle fixed in a concentration of osmium tetroxide commonly used by electron microscopist (32.7 mM OsO_4) takes up large amounts of calcium from a physiologic solution containing 1–4 mM $CaCl_2$. The mean ventricular content of calcium is increased 45-fold over that of ventricles not perfused with osmium tetroxide. A substantial part of this net calcium uptake is probably accumulated by the plasma membrane.

These observations suggest that the transverse tubular dimensions in electron micrographs of mammalian heart muscle so far published may differ greatly from the in vivo dimensions. Our experiments to date have not yet yielded a reliable value for these dimensions.

Note Added in Proof Since this manuscript was submitted, we have observed that the lumina of the transverse tubules in the rat's heart contain characteristic structures. These structures, previously described as lying in the extracellular spaces between cells at the intercalated disc (Page, E. 1967. *J. Ultrastruct. Res.* **17**:63), are delimited by three-layered membranes and may form tight junctions with one another. The significance of their presence in the transverse tubules is obscure (D. A. Meddoff and E. Page, data to be published).

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