HISTORICAL PERSPECTIVES

A perfect confluence of physiology and morphology: discovery of the transverse tubular system and inward spread of activation in skeletal muscle

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Rall JA. A perfect confluence of physiology and morphology: discovery of the transverse tubular system and inward spread of activation in skeletal muscle. Adv Physiol Educ 44: 402–413, 2020; doi:10.1152/advan.00091.2020.—By early 1954, there existed a plausible model of muscle contraction called the sliding filament model. In addition, the nature of muscle excitation was understood. Surprisingly, the link between the membrane excitation and contraction was entirely unknown. This dilemma has been called the time-distance paradox. The path to discovery of the missing link between excitation and contraction was a rocky one involving the simultaneous but independent development of physiological and morphological studies. From the viewpoint of physiology, significant events included the most thrilling moment of a scientific life, confirmation of a hypothesis that was wrong, a major surprise and shock, a result not expected from evolutionary relationships, and disappointment and confusion before clarity. From the viewpoint of morphology, there was the exciting beginning and rapid development of biological electron microscopy, heroic experiments, the importance of sample preparative procedures, and discovery of clues from the old light microscopic literature. However, it was the confluence of physiology and morphology that brought clarity and a major advance in understanding, leading to the discovery of the transverse tubular system and inward spread of activation in skeletal muscle.

Andrew F. Huxley; biological electron microscopy; Keith R. Porter; muscle activation, muscle contraction, transverse tubules

INTRODUCTION

By early 1954, there existed a plausible model of muscle contraction called the sliding filament model, where the transient formation of actin-myosin linkages induced contraction (26, 32). Also, by that time, the importance of the muscle action potential was understood. Surprisingly, the link between the membrane excitation and contraction, called excitation contraction coupling by Alexander Sandow in 1952 (61), was entirely unknown. This dilemma has been called the time-distance paradox (49).

In 1947, Heilbrunn and Wiercinsky (17) proposed that Ca$^{2+}$ was the physiological activator of muscle contraction. This proposal resulted from studies where CaCl$_2$ solutions were microinjected into frog muscle fibers. They further proposed that the Ca$^{2+}$ was stored at rest just under the muscle sarcolemma (surface membrane) and that, on activation, the Ca$^{2+}$ diffused throughout the fiber to cause contraction. In a very influential study in 1949, A.V. Hill (18) concluded, based on both theoretical and experimental results:

It is quite impossible, therefore, to explain the rapid development of full activity in a twitch by assuming that it is set up by the arrival at any point of some substance diffusing from the surface: diffusion is far too slow.

Thus diffusion of a chemical could not be the inward link to contraction. Contraction of the muscle followed the action potential after a very brief delay. If not a chemical diffusing inward from beneath the muscle surface, what then was the link between the action potential and contraction? One possibility was that the local currents set up by the action potential spread rapidly throughout the fiber to activate contraction directly. In 1946, Stephen W. Kuffler (34) showed that it was not the local current flow that activated contraction, but rather the membrane potential change. He observed that a contraction occurred with a uniform depolarization of the fiber surface by immersion in a solution of elevated potassium concentration where there would be no current flow. Another possibility was that the membrane potential change directly activated contraction. However, the membrane potential change exerted a direct influence only on the membrane itself and not on the fiber interior. Thus some other process must lead to the activation of muscle contraction.

The path to discovery of the missing link was a rocky one involving the simultaneous but independent development of physiological and morphological studies. However, it was the confluence of physiology and morphology that brought clarity and a major advance in understanding, leading to the discovery of the transverse tubular system and inward spread of activation in skeletal muscle.

Physiological Search for the Missing Link: Inward Spread of Muscle Activation

In late 1953, Andrew Huxley and Rolf Niedergerke (26) finished their famous experiments that led to the proposal of the sliding filament theory of contraction. Huxley then focused his attention on the question of how a surface action potential in a muscle fiber led to activation of contraction throughout the fiber cross section. He sought to test the hypothesis proposed in 1924 by the Australian zoologist Oscar Werner Tiegs (1897–1956) (67), who speculated that the Z line provided a pathway for the inward spread of muscle activation. By this, he meant not only the Z line as observed in a single myofibril, but also the transverse connections that linked myofibrils together and attached them to the cell membrane. Together, investigators often called this structure Krause’s membrane based on the work of the 19th century microscopist, Wilhelm Krause (1833–1910). Huxley did not envision the structure as a membrane
like the sarcolemma but rather as a solid network that in some unspecified way was a pathway for muscle activation (20, 25). Thus he started the experiments with the working hypothesis that the Z line provided a transverse pathway for the propagation of an unspecified process into the muscle fiber leading to activation of contraction.

Huxley relished this kind of experimental challenge, in part, because it involved the use of the light microscope, the instrument that would play a crucial role in his experiments throughout his career. Since his boyhood, Huxley had a long-standing interest in microscopy (7, 23) (Fig. 1). He even designed his own interference microscope. Postdoctoral fellow Robert E. Taylor, an experienced electrophysiologist from the University of Illinois, joined the Huxley laboratory to undertake the experiments. The basic plan of the experiments was to cause a local depolarization of a small area (1–2 μm) by applying a very small current, not large enough to generate an action potential, to a micropipette whose tip was in contact with the surface of an isolated muscle fiber at various spots along a sarcomere of the fiber. The hypothesis was that a local contraction would occur only when the pipette was located at the Z line.

The experiments were demanding for a number of reasons. A single muscle fiber from the frog had to be isolated. Frog muscle was the preparation of choice since it was the standard for physiological experiments in the first half of the 20th century. Glass pipettes filled with a saline solution had to have a flat tip of ~2 or 4 μm diameter and had to be placed in contact with the fiber without penetrating the fiber membrane. A micromanipulator had to be utilized that allowed very precise, submicrometer positioning of the pipette. A high-powered light microscope, at first a polarizing and later an interference microscope, was necessary for accurate identification of the fiber banding pattern and placement of the pipette. A controlled depolarizing pulse of various amplitudes had to be applied to the pipette. Finally, cine photography, at 16 frames per second, would have to be employed to observe the results of the depolarization as a function time. To improve spatial resolution, they stretched frog fibers from a normal resting sarcomere length of ~2 μm to ~3 μm.

The results of the experiments were spectacular, some of the most remarkable results in the history of muscle physiology. When they placed a 2-μm-diameter pipette opposite the A band and applied a 0.5-s depolarizing pulse, nothing happened (Fig. 2A). However, when Huxley and Taylor placed the pipette opposite the Z line, they observed a “sensitive spot” where a depolarizing pulse caused a small local contraction (Fig. 2B). The I band shortened symmetrically about the Z line. The adjacent A bands did not shorten. A hyperpolarizing pulse had no effect.

The most remarkable observation (Fig. 2C) occurred when a depolarizing pulse was applied to a 4-μm-diameter pipette placed opposite the Z line. Huxley and Taylor generated a larger depolarizing pulse with the larger diameter pipette. Under these conditions, the I band under the pipette shortened inward ~10 μm. Remarkably, this activating effect never spread longitudinally, i.e., adjacent I bands (1.5–3 μm from the central Z line) never shortened (31). Years later, looking back over his research career, Huxley (24) stated that: “The moment when I first saw this response was the most thrilling of my scientific life.” Huxley and Taylor made a fundamental discovery and confirmed their hypothesis in a most dramatic way.

In others experiments, a similar spread of contraction (up to 10 μm in each direction) also occurred along the fiber perimeter at the Z line (31). Taken together, these results suggested strongly that the inward spread of depolarization was taking place transversely, but not longitudinally, in two dimensions along some structure in the plane of the Z line.

In a letter sent to Nature in September of 1955, Huxley and Taylor (30) concluded:

We consider that these results are strong evidence that the influence of membrane depolarization is conveyed to the interior of the fibre by spread along some structure in the I band; from the anatomical situation, this must almost certainly be Krause’s membrane.

In his classic review written in early 1955, Huxley (20) was more circumspect in his conclusion:

It is not actually proved that the visible Z membrane is the structure concerned; it may be that the activation is conveyed along some other structure near the middle of each I band. The anatomical evidence ... does however show that the Z membrane has the required characteristics, and it seems most probable that it is the structure concerned.

They naturally were very pleased to have discovered a transverse pathway for inward muscle activation and to have confirmed their hypothesis that the pathway existed at the Z line. However, it turned out that there was a problem, a big problem.
Later in November 1955, Huxley and Taylor presented their results at a meeting of the Physiological Society in London (29). In the audience was a young postdoctoral fellow, J. David Robertson, who at the time was working in the Department of Anatomy at University College London. In abstracts of presentations distributed in advance of the meeting, Robertson noticed the photograph included in the Huxley and Taylor abstract of the dramatic contraction with the $4 \mu m$ pipette. As Huxley (21) later recalled, Robertson

...produced from his pocket a slide of an electron micrograph which showed unmistakable transversely oriented tubular structures in a striated muscle fibre. ...But these tubules were not at the Z level; there were two sets of them per sarcomere, one close to each end of the A band.

Robertson suggested that these tubules were the basis of the transverse spread of contraction, and that the pipettes employed by Huxley and Taylor were not fine enough to stimulate separately the tubules flanking each I band. [The slide in question subsequently appeared as figure number 10 in a paper published by Robertson in 1956 (57).] The negative result with depolarization through the $2 \mu m$ pipette placed opposite the A band (Fig. 2A) would seem to contradict this suggestion. In contrast, close examination of Fig. 2C indicates that the $4 \mu m$ pipette covered the A/I junction at each end of the I band. Thus it was possible that the depolarization traveled inward along structures located at the A/I junctions and not at the Z line. What was clear was that both the location and the nature of the inward conducting structure were now in question. This sug-
gestion must have been galling to the technical expert Andrew Huxley, not to mention that the hypothesis of Huxley and Taylor regarding Krause’s membrane in the letter already submitted to *Nature* would be invalidated. However, there was another possibility other than a technical one. Since Robertson performed his experiments on lizard skeletal muscle and not frog skeletal muscle, there might be a species difference. However, given the phylogenetic relationship of frogs to lizards, this possibility seemed unlikely (21).

Huxley (19) responded to this criticism by doing experiments on fibers from the walking leg muscles of a crab, which had a resting sarcomere length of ~8 μm instead of the 3 μm of the stretched frog fibers. Thus better spatial resolution was attainable with the pipettes previously utilized. Unexpectedly, Huxley found that the location of the sensitive positions was different from those in frog muscle. The sensitive spots along the sarcomere existed near the boundary between the A and I bands and not at the Z line. When the muscle was stimulated near the A/I junction, only the adjacent half-I band shortened and pulled the Z line toward the A band.

Huxley was disappointed that the results in crab muscle were contrary to those found in frog muscle. Under the circumstances, he felt compelled to repeat the experiments on the frog fibers, and he was able to confirm the earlier results (19). Thus there was no error in the measurements or results. He made another important observation on frog muscle fibers. When Huxley moved a pipette around the perimeter of a fiber along the Z line, responses occurred at some positions but not at others. The sensitive spots along the Z line were separated by distances of ~5 μm. This discontinuous distribution of sensitivity along a single Z line suggested that the structure in question was a network rather than a complete disk. Finally, the response to local depolarization was smoothly graded with stimulus strength, and there was no suggestion of all-or-none behavior.

To complete the picture, Ralph Straub (1928–1988) from Geneva joined the Huxley laboratory, and they (28) determined that, in lizard muscle fibers, the sensitive spots appeared at the A/I boundary and not at the Z line, just as Robertson predicted.1 The main conclusion was that the inward spread of activation could not in general be attributed to Krause’s membrane, as Huxley originally proposed.

Huxley and Taylor (31) published the work on frog and crab muscle fibers in full in 1958 wherein they concluded:

> The simplest hypothesis consistent with our results is that each fibre contains networks of tubules, electrically continuous with the external fluid, in the transverse planes corresponding to the positions at which surface depolarization is effective (Z line in frog; near to boundaries of A band in crab), and that reduction of the potential difference across the walls of these tubules activates the neighbouring myofibrils.

Thus after some frustration and disappointment, the “thrilling” observation of the localized inward spread of muscle activation was determined to be genuine, but the picture was confusing. The sensitive spots occurred in different parts of the sarcomere in different muscle species. A no doubt frustrated Andrew Huxley (21) noted that this is “...not at all what might have been expected from phylogenetic relationships.” Indeed, further confusion would result from the crab muscle experiments before clarity would be achieved (see below). Clearly, investigators required a deeper structural understanding of the putative internal membrane structure of muscle fibers. It turned out that this understanding also was evolving in the 1950s.

**Development of Biological Electron Microscopy and Discovery of the Sarcoplasmic Reticulum and Transverse Tubules**

The first electron micrograph of an intact animal cell appeared in 1945 (53) and marked the beginning of electron microscopy in cell biology. Keith R. Porter* and Albert Claude at the Rockefeller Institute for Medical Research collaborated with electron microscope technician Earnest Fullam at the International Corporation of Manhattan, an industrial laboratory.3 The image that they produced was of a fibroblast-like cell cultured from chick embryo tissue. The magnification was a modest ×1,600. Looking back on it, Porter admitted that the images were not much better than the light microscope images (39). Still, it was thrilling all the same. In 1970, Porter (39) said

> ...It was wonderful, believe me, we had never seen anything like it. Men have visited the moon...but we were the first...to see particles, to see structures that the light microscope had not been able to resolve.

Excitement aside, a problem was the thickness of the cultured cell. Near the thinner periphery of the cell, they observed in the ground substance what they called a “lace-like reticulum.” Porter went on to characterize this reticulum in the late 1940s and 1950s and named the structure the endoplasmic reticulum. Nonetheless, there was a need for producing thin sections of the preparations.

There was the very real possibility that some of the observed structures were artifacts of tissue preparation. In the early 1950s, there was a major effort to improve the fixation procedure and to develop thin sectioning techniques appropriate for electron microscopy. A fixative must change the chemical composition of the cell in such a way as to confer structural

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1 J. David Robertson (1923–1995), who became an eminent electron microscopist, felt that he never received the credit that he deserved for suggesting the role of tubules in the inward spread of muscle activation. Robertson has told the story this way (58). In 1954, he made presentations of his ideas in the Rockefeller Institute for Medical Research did not have its own electron microscope (39).

2 Many investigators consider Keith Roberts Porter (1912–1997) to be the “father” of cell biology. Porter was a member of the National Academy of Sciences and won many awards, except one. As summed up by Manfred Schlüwa (62): “Not surprisingly, as someone who accomplished so much during his 50-year scientific career, Porter also received numerous awards and prizes, except one. Indeed, many of us believe that he should have been among those who received the Nobel Prize for contributions to cell biology.” For a description of Porter’s research career, see Peachey and Brinkley (45).

3 It is ironic that Porter and Claude had to go to an industrial laboratory to do the first electron microscope experiments on an intact animal cell because the Rockefeller Institute for Medical Research did not have its own electron microscope (39).
stability without causing major distortion of cellular structure. In 1927 Straneways and Canti (66) found that osmium tetroxide (OsO\textsubscript{4}), a fixative employed since the 1860s (6), produced almost perfect preservation of the internal structure of cultured cells for observation with darkfield microscopy. Of course, they had the advantage of looking at the cells before and after fixation. The electron microscopist did not have that advantage. Nonetheless, Porter felt that their osmium tetroxide fixation was the most satisfactory for electron microscopy “in that it yields what impresses one as being the most truthful picture of the cell” (53). In 1952, George E. Palade (42) improved on this procedure by buffering the 1 or 2% osmium tetroxide solution at physiological pH. This fixative became affectionately known as “Palade’s pickle” (39) and was the standard primary fixative for a decade (see below). The idea was that osmium tetroxide worked by decomposing and leaching out the protein matrix surrounding the formed elements of the cell, leaving behind a membrane skeleton that was excellent for electron microscopy (39).

There was a critical need for the generation of reproducible cell sections thin enough for the electron beam to penetrate. These ultrathin sections needed to be ~0.1 μm thick (up to 100 times thinner than sections for light microscopy). A myriad of microtomes appeared in the 1950s for this purpose. In 1953, Porter and Rockefeller instrument maker Josef Blum developed a popular microtome, Porter-Blum microtome (52). It was a superior, reliable, and easy-to-use instrument. Rapid progress occurred in the quality of the electron microscope images with the development of the Porter-Blum microtome, glass knives, buffered osmium tetroxide as a fixative, and plastic embedding media. For a brief history of these developments in early biological electron microscopy, see Pease and Porter (50).

The first electron microscopic study of thin sections of skeletal muscle was performed by H. Stanley Bennett (1911–1992) and Porter in 1953 (5). Among other observations in this exploratory study of breast skeletal muscles of the fowl, they described an intracellular membrane system. The membrane system exhibited longitudinal and transverse components. The longitudinal component represented “sections through braces or encircling rings of reticulum which encompass each myofibril.” The transverse component of the reticulum existed as “diffuse reticulum connections between Z and sarcolemma.” They named the structure the sarcoplasmic reticulum (SR) in analogy to the endoplasmic reticulum discovered and characterized by Porter in other cell types. They commented that their results confirmed “to a remarkable degree” the relationships and structures recognized by the 19th century light microscopists.

With the advances in preparative techniques and in the quality of journal reproduction of electron micrographs4, in 1957 Porter and Palade (54) made a comprehensive study of the SR. They examined the SR of amphibious muscle (myotome fibers of Amblystoma larvae) and rat skeletal and cardiac muscle. Myotomes of Amblystoma larvae received the major attention because they showed the reticulum to “excellent advantage.” They observed a membrane delineated intracellular system with both longitudinal and transverse components.

They concluded that:

... an elaborate lacework or reticulum of tubular and vesicular elements exists as a structural component of the interfibrillar sarcoplasm. This reticular component is essentially continuous and disposed in such a way that if the myofibrils were removed, it would appear as a fine honeycomb. For convenience of consideration it may be regarded as forming a sleeve around each myofibril, though obviously parts of any one sleeve are shared with surrounding myofibrils.

At the level of the Z line in amphibian muscle, the reticulum became dilated. The dilated portion of one sarcomere faced an equivalent dilated portion in the next sarcomere. Porter and Palade (54) called the dilated portions of the SR terminal cisternae. A space of ~500 Å existed between the dilated pairs of the SR. This space contained a single row of profiles of small vesicles running transversely across the fiber. The two opposing dilated units (terminal cisternae) and the intervening space, containing small vesicular units, constituted a three-component structure that they named a “triad.” Thus one triadic network existed at the level of each Z line in amphibian muscle. Figure 3 shows a schematic illustration of the SR as interpreted by Porter and Palade.

Ebba Andersson-Cedergren (1), a graduate student, working in Fritiof S. Sjostrand’s laboratory in the Department of Anatomy at the Karolinska Institute in Stockholm, made an important conceptual advance in 1959. In what has been called a heroic study (13), Andersson-Cedergren (1924–1974) examined the SR in serial sections of mouse muscle fibers. She noted:

One part of this network consists of transversely oriented tubules which may appear as rows of small vesicles. This part extends from the plasma membrane across the muscle fiber close to the level of the A-I boundary and appears to form a transversely oriented system, which is not continuous with the rest of the sarcotubular system. This system will here be referred to as the transversal or T system. ... The middle component of the triad, the T system, consists of elements which in longitudinal sections usually look like fragments of tubules or vesicles. However, in cross sections through the muscle fibers the T system consists of tubules with a very complicated convoluted shape. ... The three dimensional reconstruction has clarified this relation. ... The convoluted tubules appear to form continuous components extending over long distances across the muscle fiber. This conclusion is based on the observation that the tubules can be traced throughout the series of sections with few exceptions.

Whereas the transverse tubular system of the T system displayed intimate contact with the plasma membrane, it was not possible to observe any continuity of the tubular walls and the plasma membrane. Thus there was no indication that the lumen of the tubules communicated directly with the extracellular space.

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4 Porter and Bennett were greatly disappointed in the quality of the halftones of electron micrographs that appeared in their 1953 paper. They along with George Palade felt that there was a need for a new interdisciplinary journal with editorial policies and technical capabilities appropriate for the developing field of cell biology (39, 51). Rockefeller Institute Director Detlev Bronk supported the request with the proviso that the journal not be limited to electron microscopy. Furthermore, he agreed that the Institute would cover the costs, and that the Rockefeller Institute Press would produce the journal. After much discussion, the journal with the cumbersome name Journal of Biophysical and Biochemical Cytology appeared in 1955. Porter never liked the name, but it was a compromise to get it produced. The journal soon changed its name to the now famous Journal of Cell Biology in 1962.
Thus by 1959, it was realized that there was not one intracellular membrane system but rather two discontinuous membrane systems (1). One component was the SR, and the other the T system. The T system was a complicated tubular system running across the muscle fiber and not a series of small vesicles, as suggested by Porter and Palade. These days, these tubules are referred to as transverse tubules or T tubules. Finally, the triads in the mouse fibers did not exist at the Z line, as they did in amphibian fibers, but existed at the boundary between the A and I bands. Whether or not the T tubules communicated directly with the extracellular space was an open question.

Function of the Transverse Tubules: A Confluence of Physiology and Morphology

Bennett (2) proposed in late 1954 that the SR might “function to conduct an excitatory impulse from the periphery of the cell . . . to myofibrils in the interior so that contraction of all myofibrils across the diameter of the fibril is well-coordinated.” This hypothesis became far more attractive when Huxley and Taylor (31) and Huxley and Straub (28) showed the locations of the inward spread of activation in frog, crab, and lizard muscle.

Bennett (3) has stated that the he and Porter (54) rediscovered the SR with the electron microscope. He was very respectful of the old light microscopic literature on muscle. Bennett felt that the most meticulous and convincing study of the reticular apparatus of striated muscle was the one conducted by Emilio Veratti (1872–1967) (68) at the University of Pavia in 1902. Bennett (3) stated, “This paper must be recognized as one of the most important ever published on muscle structure.” He went on to state: “It is astonishing that a structure once described as accurately and as beautifully as the reticulum was by Veratti (1902), should have so quickly become almost lost to man’s knowledge” (4). So enthusiastic was Bennett about Veratti’s results that he helped translate the paper from Italian and had it republished in a special issue of the Journal of Biophysical and Biochemical Cytology in 1961 (69), along with a collection of other papers on the SR. What did Veratti observe in this massive paper with 41 drawings? Using the “black reaction” (precipitation of silver chromate) discovered by Camillo Golgi in 1873 (the Golgi stain) (35), Veratti observed in the light microscope transverse and longitudinal structures in fibers of a vast array of muscle types. As beautiful and impressive as this work was, it was forgotten completely along with the other early studies until the rediscovery of the SR in the 1950s using electron microscopy. One wonders why it was forgotten, Possibly Smith (64) in his review of the 19th century light microscopic literature on the muscle reticular system said it best: “. . . at the turn of the century there was scarcely a single aspect of muscle organization that was not entirely controversial.”

In 1960, Bennett (4) made the astute connection between Veratti’s observations and those of Huxley, Taylor, and Straub, and the confluence of physiology and morphology began in earnest. Veratti’s results regarding transverse structures in muscle fibers dovetailed exactly with the location of the sensitive spots in the sarcomeres of frog (Fig. 4, top) and lizard muscle (Fig. 4, bottom) as seen by Huxley, Taylor, and Straub. This structural information helped greatly in interpreting and reinforcing the physiological data. Based on the structural studies, the sensitive spots likely represented places in the sarcomere where the T tubules were located. Veratti’s results and the electron microscopic studies of Porter and Palade (54) on amphibian muscle and Andersson-Cedergren (1) on mouse muscle were compatible with this proposal. Thus the T tubules might convey the surface depolarization into the muscle fiber, leading to contraction.

The pieces were starting to come together but unfortunately not all of the pieces. There was a major complication. Lee D. Peachey, a graduate student working in Porter’s laboratory, came to work with Huxley in 1958 (44).6 While there, he undertook various studies with Huxley, including an electron

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5 Several observers in the 19th century reported reticular networks inside muscle cells, but, in retrospect, it was difficult to judge whether these early observations were of a true internal reticulum or coagulated sarcoplasm because of the variable and controversial methods of tissue preparation (64). To confuse matters even more, in the 1880s, some investigators proposed that the myofibrils were artifacts of fixation, and that the internal reticulum was the contractile component of the muscle cell (23).

6 In the early 1950s, the Rockefeller Institute for Medical Research appointed Detlev Bronk, former president of Johns Hopkins University, as the Director (39). His vision was to transition the Institute into a PhD granting graduate university, and the Rockefeller University began. Lee D. Peachey (b. 1932) was among the first group of graduate students at the new university. Included in Bronk’s vision was that the graduate students should obtain an
microscopic study of crab skeletal muscle. Their results were a
disappointment to both Huxley and Peachey. They were able to
identify transversely oriented tubules at the Z lines of the crab
muscle, but not at the A bands (21). The physiological results
(31) showed just the opposite, sensitive spots were located at
the A band and not the Z line! Clearly, something was wrong.
Was it the electron microscopic results, the physiological
results, or the general hypothesis of inward activation?
The problem turned out to be a technical one related to the
fixation process for electron microscopy. In the early 1960s,
Russell Barrnett at Yale University was exploring the applica-
tion of histochemical techniques to electron microscopy. He
was having a problem because the standard fixative osmium
tetroxide irreversibly inactivated the enzymatic activities that
he wanted to relate to subcellular structures. Because David D.
Sabatini7, a new investigator in the laboratory, was the most
experienced electron microscopist in the laboratory at the time,
he was assigned the task of looking for an alternative fixative.
His very first experiments showed that glutaraldehyde, a good
cross-linking reagent, gave an excellent preservation of cellular
structures while allowing the demonstration in situ of several
specific enzymatic activities. The resulting paper in 1963
caus[ed] an immediate change in the primary fixative procedure
for electron microscopy (60). The paper has been cited over
4,000 times.8

After a brief interlude to share the 1963 Nobel Prize with
Alan Hodgkin and Sir John Eccles, Huxley returned to the crab
skeletal muscle experiments with Peachey, now with the new
fixative in hand. First, they confirmed the physiological results
(27). Half-sarcomere contractions were observed by depolar-
zizing a region over the outer parts of the A bands, but no
contractions were elicited by depolarization of regions of the
membrane over the Z lines. Second, with glutaraldehyde fix-
atation, once again they observed transversely oriented tubules at
the Z line but now also clearly saw transverse tubules located
near A/I junctions (48). Unlike in other muscles, the tubules
near the A/I junctions formed “dyadic” associations, rather
than triadic associations, with the longitudinal SR. The tubules
at the Z line did not form any associations with SR. It turned
out that the association of the transverse tubules with the SR
would make all the difference from the viewpoint of muscle
activation (see below).

Thus the T system of a muscle cell became defined as (47)
“a network or a series of networks of tubular invaginations (T
tubules) of the plasma membrane of the cell that forms specific,
functional associations with the SR.”9 According to this defi-

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8 Osmium tetroxide, first employed as a fixative in light microscopy over
150 yr ago (6), often is employed still as a secondary fixative and as an agent
to enhance contrast.

9 Across the animal kingdom, there is a variation in the location of triads in
the sarcomere (65). In both fish and amphibian skeletal muscle, Z line triads
have been described, whereas A/I triads occur in some fish, birds, reptiles, and
mammals. In invertebrate muscle fibers, the location of triads, dyads, and
peripheral couplings is more variable, with a complete range from fixed to a
random positioning. For example (40), some small-diameter muscle fibers that
contain only one myofibril and are ~1 µm thick, like that adductor striated

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Fig. 4. A schematic representation of the spots in muscle fibers sensitive to local depolarization compared with Veratti’s drawings of the muscle reticulum. Top:
frog fiber, with the micropipette at the Z line, before (1) and after (2) local depolarization compared with Veratti’s drawing (3) of the reticulum in frog muscle.
The blackened strands of the reticulum lie along the Z lines precisely where Huxley and Taylor (31) found the spots sensitive to local depolarization. Bottom:
lizard fiber, with the micropipette at the A/I boundary, before (4) and after (5) local depolarization compared with Veratti’s drawing (6) of the reticulum in lizard
muscle. The blackened strands of the reticulum lay along the A/I boundaries exactly where Huxley and Straub (28) found the spots sensitive to local depolarization. [From Mazzarello et al. (37) with permission.]

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In 1961, David D. Sabatini (b. 1931), an MD from Argentina, wanted to go
to the Rockefeller University to work for a PhD with George Palade (59).
However, because of a temporary space limitation, Palade suggested that
Sabatini go to Yale University for a few months to work with Russell Barrnett,
a well-known histochemist.
nition, the transversely oriented tubule at the Z line of crab muscle is not a T tubule. It is called a Z tubule to avoid confusion. Its function remains unknown.

Figure 5 displays a classic representation of the SR and T tubules and their association with each other in frog skeletal muscle (43). The T tubules represented ~0.3% of the fiber volume and the SR ~4–5% of the fiber volume. Even so, their combined surface area was 50–60 times greater than the outer surface area of a 100-μm-diameter fiber.

Further Elucidation of the Transverse Tubular System and its Association with the Sarcoplasmic Reticulum

A further characterization of the T system came with the introduction of high-voltage electron microscopy (HVEM). In conventional transmission electron microscopy, the sections had to be ultrathin, ~0.1 μm thick, to allow for penetration of the electron beam at accelerating voltages of 80 kV or less. With HVEM, the microscope operated at 1,000–3,000 kV, and these high voltages allowed for the examination of much thicker sections in the range of 3 μm. Peachey and Eisenberg (46), using a peroxidase stain selective for T tubules, examined serial transverse sections in HVEM. They reconstructed an entire network of T tubules in a frog muscle fiber as projected onto the transverse plane (Fig. 6, left). The T tubules surrounded gaps through which the myofibrils would run. The complexity is amazing. One can imagine a Huxley and Taylor local depolarization experiment, where the local activation spread both inward and laterally along the T tubules. Veratti (69) saw essentially the same structure in his cross sections of various muscle fibers (12).

In Peachey’s (43) classic electron microscopic study of the SR and T tubules in frog muscle, he occasionally observed longitudinally oriented tubules that interconnected successive networks of T tubules. Over time, it became clear that Veratti (69) did not detect the SR as defined today due to the inherent limitation of the light microscopic technique. Rather, he saw only T tubules (12). Thus Bennett was wrong in this regard. This conclusion was important because almost all of Veratti’s drawings of the muscle reticulum showed both transverse and longitudinal components. For example, see the Veratti drawing in Fig. 4, top right. This result had to mean that successive networks of T tubules also exhibited longitudinal connections. Figure 6, right, shows an example of a network of T tubules with both transverse and longitudinal components (41). This view of a section 1–1.5 μm thick with HVEM displays tubules of human skeletal muscle impregnated with lanthanum. As in mammalian muscle in general, the T tubules appear at the A/I

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Fig. 5. The sarcoplasmic reticulum (SR) and transverse tubules (T tubules) of a frog muscle fiber. Left: longitudinal electron micrograph showing the SR and T tubules in relation to myofibrils. The myofibril underlying the SR is in register with the myofibril at the right side of the figure that provides a reference to the myofibril bands. Triads appear at the top and bottom of the figure, opposite Z lines. Right: three-dimensional reconstruction of the SR and T tubules associated with several myofibrils in a muscle fiber. [From Peachey (43) with permission.]
junctons in the sarcomere. However, it also is clear that some
lubules are longitudinally oriented, connecting T tubules that
are transversely oriented. Recent estimates of the fraction of
lubules that are longitudinally oriented in a T system range
from 2 to 5% in amphibian and mammalian skeletal muscle,
respectively (33).

Thus the T system contains both transversely and longitu-
dinally oriented tubules. What do the longitudinal tubules do?
Previously, investigators thought that there were one or two
independent T tubular networks in each sarcomere along the
length of a muscle fiber, but the longitudinal connections
suggest that the entire T system of a muscle fiber may be one
continuous unit (47). This structure could provide a safety
factor that ensures full activation of myofibrils in situations
where an obstruction of sarcolemmal excitation occurs. An-
other possibility stems from the realization that the T tubules
initially form as longitudinal tubules that rearrange into trans-
verse networks as the fiber develops (69). The longitudinal
connections of T tubule networks may be remnants of the
initial longitudinally oriented networks (12). Most importantly,
they do not alter our understanding of T system function,
which is the transmission of the electrical event from the fiber
periphery into interior.

In a symposium held to honor the 100th anniversary of
Veratti’s classic paper of 1902, Franzini-Armstrong (12) com-
mented that Veratti’s drawings showed the system of T tubules
with “exquisite accuracy.” She felt that it was “quite extraor-
dinary” that he could capture images of such detail with the
light microscope. She went on to express the view that: “In
comparison with this feat, all that electron microscopy has
achieved, as regards the distribution of transverse tubules, is
essentially to confirm Veratti’s work.” Quite a compliment for
research that investigators ignored before Bennett discovered it
in the 1950s. Nonetheless, Veratti did not propose any
function for the structures that he observed. He lived to see the
revitalization of his work in the 1960s but never quite under-
stood what all the fuss was about (35).

Are the Transverse Tubules Open to the Surface?

One of the problems in answering this question was that the
T tubules often made a convoluted approach to the surface
membrane of a fiber, and it was not possible to be sure that they
were open to the extracellular space. A flurry of structural and
functional studies that appeared in 1964 proved that the T
lubules are open to the surface and are likely invaginations of
the surface membrane. Most convincing was the study of
Franzini-Armstrong (b. 1938) and Porter (15). They provided
electron microscopic evidence that the T tubules were invagi-
nations of the surface membrane in fish striated muscle, where
the T tubules made a straightforward approach to the surface
membrane. They utilized glutaraldehyde fixation to fix muscles
of the Black Mollie, a small fish 2–3 cm in length. They did
dsomething that seems at first glance very strange. To avoid
anesthesia and dissection, they immersed the whole animal in
the fixative for 1.5 h! Strange as it may seem, their results were
beautiful and convincing (Fig. 7, left). They concluded that:
“The T system is a sarcolemmal derivative that retains its
continuity with the sarcolemma and limits a space that is in
direct communication with the extracellular environment.”
Furthermore, they observed ~20–30 T tubules at each Z line
level around the circumference of the fish fibers. This result
was reminiscent of the circumferential separation of the sen-
sitive spots at the Z line in frog fibers (31). The scanning
electron micrograph in Fig. 7, right, shows a dramatic confir-
mation that T tubules open to the surface of a frog muscle fiber
(35). Openings on the surface occurred at the Z line and
circumferentially around the fiber at the Z line just as predicted
by the electrophysiological experiments of Huxley and Taylor. Thus the T tubules are invaginations of the surface membrane.

These experiments also reinforce the conclusion that the T system is separate from the SR. The T system is an invagination of the surface membrane, whereas the SR exists totally within the fiber. Certainly, they are in close apposition at the triad, but is there any connection between the two systems? Clara Franzini-Armstrong (11) showed that there were structures that reached across the 100- to 150-Å gap from the SR to the T system. At periodic intervals of ~300 Å, the SR “membrane forms small projections, whose tips are joined to the T system membrane by some amorphous material” (Fig. 8). She called these processes “SR feet.” Over time, some investigators have whimsically called these foot processes “Clara’s feet.” At the time of their discovery, the functional significance of these structures was a mystery, but they soon would become important in understanding the excitation-contraction (E-C) coupling process (see Ref. 13).

**Is the Spread of Activation into the Transverse Tubular System Active or Passive?**

Huxley and Taylor (31) interpreted their results to indicate that inward spread of contraction into the muscle fiber was due to a passive electronic spread of depolarization along the T tubules. They based this interpretation on the observation that the distance to which a contraction spreads inward from the fiber surface varied in a graded manner, according to the strength of the applied pulse. Nonetheless, they still considered the mechanism of inward spread to be an open question. The other possibility was a regenerative depolarization in the tubular membrane. Hugo Gonzales-Serratos (d. 2011) came to Huxley’s laboratory from Mexico in the early 1960s to work for a PhD. He devised a remarkable experiment to determine the velocity of inward spread of contraction in a single frog muscle fiber.

![Fig. 7. Transverse tubules (T tubules) are invaginations of the surface of a muscle fiber. Left: longitudinal electron micrographs of a T tubule in fish muscle fibers. Note that the T tubule is located at the Z line, opens to the surface, and exhibits a close relationship with the sarcoplasmic reticulum [From Franzini-Armstrong and Porter (15) with permission.]. Right: longitudinal scanning electron micrograph of the surface of a frog muscle fiber. Arrows indicate parallel rows of openings corresponding to the T tubules. The openings are found at the level of the I band near the Z line and are distributed circumferentially around the fiber.10 [From McCallister and Hadek (38) with permission.]

![Fig. 8. Structure of the triad in a frog muscle fiber. Electron micrographs showing periodic structures connecting the T tubule to the sarcoplasmic reticulum. Franzini-Armstrong has called these structures “SR feet.” [From Franzini-Armstrong (11) with permission.]](https://example.com)

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10 Whereas the locations of the T tubule openings on the surface of a frog muscle fiber are clearly shown in this scanning electron micrograph, the estimated diameters of the openings (100–200 nm) are 5–10 times larger than those observed in transmission electron micrographs of well-fixed material (14). Apparently this discrepancy is due to the unavoidable shrinkage in the dehydrated muscle that was air-dried for 24 h. The visible openings may be sites where the shrinking T tubules pull on the plasma membrane, producing cones that identify the sites of T tubule plasma membrane connections, although not the actual openings themselves.
Conclusions

The current understanding of the role of the T system in muscle activation required the sustained focus and contributions of many scientists over a number of decades. This story was exciting, sometimes thrilling, and often perplexing. It involved some of the giants of biological science and the once forgotten past. Ultimately, the confluence of physiology and morphology led to the solution of the time distance paradox of E-C coupling in skeletal muscle.

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Author Contributions

J.A.R. conceived and designed research; analyzed data; prepared figures; drafted manuscript; edited and revised manuscript; approved final version of manuscript.

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A Further Synthesis

Thus the first steps of E-C coupling, as defined by Alexander Sando (61), were elucidated both functionally and structurally. What was missing in 1958 was an understanding of what happened next. Huxley and Taylor (31) summed up the situation: “There is little evidence concerning the final step from these hypothetical transverse networks to the filaments themselves.” It was soon thereafter that biochemistry combined with physiology and morphology to further advance the understanding of E-C coupling. Micromolar concentrations of Ca2+ were shown to regulate the ATPase activity of actomyosin (9, 70) and a vesicular relaxing factor isolated from muscle that became known as the SR was shown to exhibit an ATP-dependent binding of Ca2+ (9). In 1961, Setsuro Ebashi (9) combined the evidence for Ca2+ as the physiological activator of contraction (17), the structural evidence of the SR (54), and the evidence of the inward spread of activation (31) with the biochemical evidence into a coherent hypothesis for the role of Ca2+ in muscle contraction and relaxation that has guided research ever since. For a contemporary review of the historical development of E-C coupling, see Fratzani-Armstrong (13).
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