Chapter 4
Excitation-Contraction Coupling and the Role of Calcium in Contraction and Relaxation in the 1950s and 1960s

We shall designate the entire sequence of reactions-excitation, inward acting link, and activation of contraction-by the term excitation-contraction (E-C) coupling. (Sandow 1952. With permission Yale Journal of Biology and Medicine)

Alexander Sandow (1952)

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. (Hill 1949. With permission The Royal Society)

A. V. Hill (1949)

It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation. (Ebashi and Endo 1968. With permission Elsevier)

Setsuro Ebashi and Makoto Endo (1968)

4.1 Introduction

While spectacular advances were being made in the understanding of the mechanism of contraction during the 1950s and 1960s, there were equally exciting advances during this same period in the understanding of how muscles were turned on and turned off. Alexander Sandow at New York University gave a name to this new field of study of activation of muscle contraction when he coined the term "excitation-contraction coupling" (Sandow 1952). He defined the field this way: "We shall designate the entire sequence of reactions—excitation, inward acting link, and activation of contraction—by the term excitation-contraction (E-C) coupling". The elucidation of the fundamental features of E-C coupling came from a melting of physiology, cell biology and biochemistry. Even though this is a great success story, there were mis-steps, forgotten clues and strong personalities along the way. And as pointed out by Ebashi and Endo (1968): "It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation." This period also resulted in the emergence of the influence of Japanese scientists in the muscle field. This emergence was almost single handedly due to the outstanding achievements and influence of Setsuro Ebashi.

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4.2 Muscle Excitation and Onset of Contraction

At the time when Alan Hodgkin and Andrew Huxley were doing their classical experiments elucidating the ionic mechanism of the nerve action potential in 1949, William (Bill) L. Nastuk came to the Hodgkin laboratory from Columbia University to work on the excitation of skeletal muscle. In 1948 Hodgkin had visited Ralph Waldo Gerard's (1900–1974) laboratory at the University of Chicago where he learned of the possibility of making intracellular electrical recordings with microelectrodes. While there he met a Chinese investigator, Gilbert Ling, who had found that if he drew a 1 mm glass tube down to a fine tip, it would penetrate a frog muscle fiber easily, giving a stable resting potential while causing no damage to the fiber. The trick was that the tip had to be so fine that it disappeared when looked at with visible light under a high-powered objective (Hodgkin 1992).

Gerard had the original idea to develop the microelectrode. Judith Graham produced microelectrodes with tip diameters of several micrometers and they used these larger microelectrodes to measure resting membrane potentials in skeletal muscle fibers (Graham and Gerard 1946). Gilbert Ling perfected the technique by making smaller microelectrode tips of about 0.5 μm diameter which led to measurement of the first accurate values of the resting membrane potentials in frog skeletal muscle (Ling and Gerard 1949). Ling taught Hodgkin how to make and fill these microelectrodes with a potassium chloride solution for which Hodgkin was "greatly indebted" (Nastuk and Hodgkin 1950). Ling and Gerard gave Hodgkin permission to use the microelectrodes in an attempt to measure action potentials in skeletal muscles back in Cambridge. Hodgkin (1992) was particularly interested in these experiments because they helped to answer the criticisms that the work on crab or squid nerve might not be relevant to mammalian excitable tissues. What resulted (Nastuk and Hodgkin 1950) were the first intercellular recordings of action potentials in skeletal muscle.

The action potentials observed in skeletal muscle fibers of the frog with microelectrodes of about 0.5 μm tip diameter were qualitatively similar to those observed in squid axons. The action potential started from an average resting potential of −88 mV and overshot the zero potential by about 30 mV. The magnitude of the action potential was strongly depressed when the extracellular Na^+ concentration was decreased whereas little change was observed in the resting membrane potential. These results were similar to those observed in squid axons and supported the generality of the theory that the action potential was due to a specific increase in membrane Na^+ permeability.

There were some differences in the muscle action potentials compared to the previously observed nerve action potentials. Most notable was the tail or slow repolarization of the action potential, called the negative after-potential, that was always seen in the skeletal muscle action potentials but not in the squid axon action potentials. Another notable difference was that the capacitance of the skeletal muscle fiber membrane was found to be about five times greater than that observed in nerve fibers (Katz 1948). Membrane capacitance is a function of the chemical structure of the membrane and is expressed relative to the area of membrane as micro-farads per centimeter squared ($\mu F/cm^2$). Thus either the muscle membrane was fundamentally

different from the nerve membrane, which seemed unlikely given the similar nature of the action potential, or somehow the area of the muscle membrane was substantially under estimated when based on fiber diameter alone. It would be some years before this mystery was solved in favor of the latter possibility.

Contraction of the muscle follows the action potential after a very brief delay. In a very influential study in 1949, A. V. Hill showed by stretching a frog muscle rapidly immediately after an action potential that it reached peak active force within 40 ms at 0 °C. Hill (1948) estimated the time that it would take for any putative activator substance to diffuse from the cell membrane to the center of a typical frog muscle fiber of 100 μm diameter. Based on this calculation, Hill (1949) concluded that "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." The situation is even more acute when a muscle contracts at a higher temperature since diffusion is relatively temperature insensitive. For example, the interval between the action potential and the start of contraction which is called the latent period is about 6 ms in a frog fiber at 20 °C (Buchthal and Sten-Knudsen 1959).

If not a chemical diffusing inward from the muscle surface, what then is the link between the action potential and contraction? One possibility is that the local currents setup by the action potential could spread rapidly throughout the fiber to directly activate contraction. Kuffler (1946) showed that it is not the local current flow that activates contraction but rather the membrane potential change. He observed that a contraction is produced even by a uniform depolarization of the fiber surface by immersion in a solution of elevated K^+ concentration where there should be no current flow. This result was confirmed by Watanabe (1958) who passed current between two intracellular microelectrodes and found that no contraction occurred. Another possibility was that the membrane potential change could directly cause contraction. But the membrane potential change would be expected to only exert a direct influence on the membrane itself and not propagate into the fiber interior. Thus there must be some other process that leads to the activation of a muscle fiber. In early 1950s this process was totally unknown.

4.3 Inward Spread of Muscle Activation

In late 1953, Andrew Huxley and Rolf Niedergerke were finishing up their famous experiments which led to the development of the sliding filament theory of contraction. At about this time Robert (Bob) E. Taylor[1] joined the Huxley laboratory from

[1] Robert E. Taylor received his Ph.D. from the University of Rochester. After a brief stay in R. W. Gerard's laboratory at the University of Chicago, he went to Andrew Huxley's laboratory from the University of Illinois College of Medicine in Chicago. He then went to the Laboratory of Biophysics at the National Institutes of Health in Bethesda, Maryland in 1956 where he spent his research career. Besides doing experiments at the NIH, he also worked at the Marine Biological Laboratory at Woods Hole for many years. He investigated electrophysiological properties of nerve fibers. In 1982 Bezanilla, White and Taylor discovered the gating current associated with the activation of K^+ channels.

the University of Illinois (Chicago). Huxley and Taylor worked together to produce some of the most famous experiments in the muscle field which established the mode of the inward spread of muscle activation. There were mis-steps and surprises along the way, starting with a hypothesis that turned out to be wrong. In 1924 the Australian zoologist Oscar Werner Tiegs (1897–1956) proposed that the Z line could provide a pathway for the inward spread of muscle activation. By this he meant not only the Z line as observed in a myofibril but also the transverse connections that linked myofibrils together and attached them to the cell membrane. Together this structure was called Krause's membrane based on the work of the nineteenth century light microscopist, Wilhelm Krause (1833–1910). It was not envisioned by Huxley and Taylor as a membrane in the usual sense but rather as solid network that might in some unspecified way be a pathway for muscle activation (Huxley 1995). Thus they started their experiments with the working hypothesis that the Z line provided a transverse pathway into the muscle fiber leading to activation of contraction.

But how does one test this hypothesis? The immediate answer is: with great difficulty. This difficulty was exactly the kind of experimental challenge that Andrew Huxley relished. The basic idea was to cause a local depolarization by applying a very small current, not enough to generate an action potential, to a micropipette whose tip was in contact with the surface of a muscle fiber at various spots along a sarcomere of the fiber. The prediction was that a local contraction would occur only when the pipette was at the Z line. This experiment was demanding for a number of reasons. A single muscle fiber from the frog had to be dissected. The glass pipette, of the Ling and Gerard (1949) type, filled with a saline solution had to have a flat tip of diameter no more than 2–4 μm and had to be placed in contact with the fiber but not penetrate the fiber membrane. A micromanipulator would have 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 magnitudes 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 resolution, the frog fibers were stretched from a normal resting sarcomere length of about 2.2 μm to about 3.0 μm.

The results of the experiments were spectacular (Huxley and Taylor 1958). When the pipette was placed opposite the A band and a depolarizing pulse applied, nothing happened (Fig. 4.1a). But when the pipette was placed opposite the Z line, a "sensitive spot" was observed where a depolarizing pulse caused a local contraction (Fig. 4.1b). The I band shortened symmetrically about the Z line. The adjacent A bands did not shorten. A hyperpolarizing pulse had no effect. Most remarkably (Fig. 4.1c), with a larger pulse the I band to which this pipette was applied shortened about 10 μm inward from the surface while the adjacent I bands (1.5–3 μm from the central Z line) did not shorten but rather were passively stretched (Huxley and Taylor 1955a). It was as if some structure directed the depolarization transversely into the fiber but not longitudinally. In a paper sent to Nature in September of 1955,

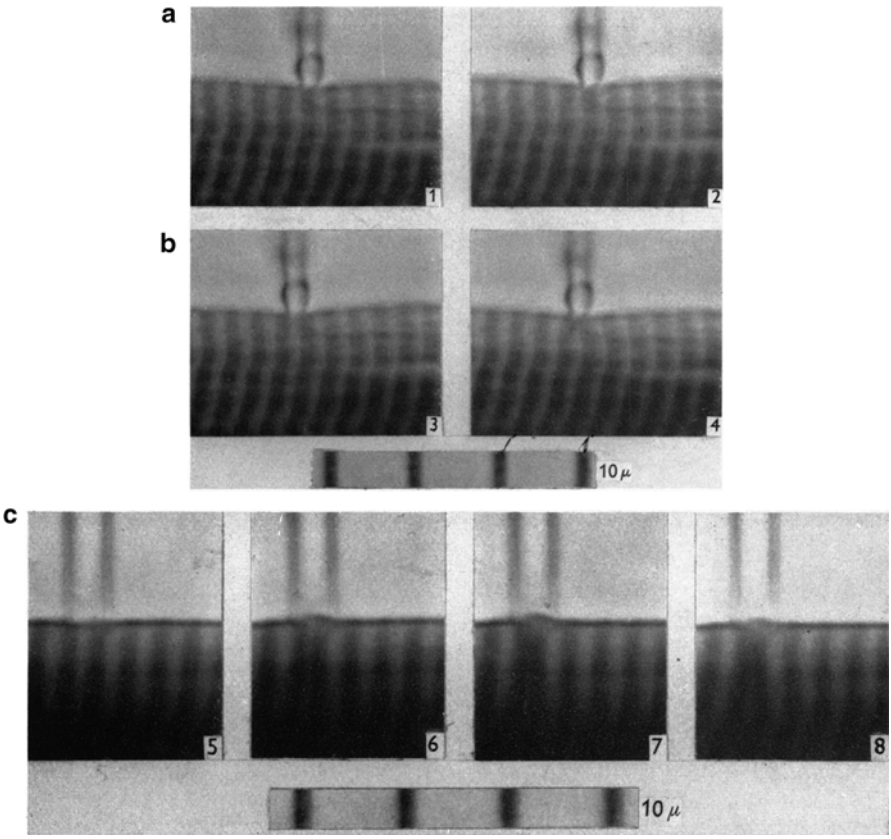

Fig. 4.1 Photographs of an isolated frog muscle fiber in contact with a micropipette. Polarized light compensated so that the A bands appear dark. (**a**) The pipette (tip diameter 2 **μ**m) is applied in images 1 and 2 to an A band and (**b**) in images 3 and 4 to an I band. In each case, the left-hand picture is taken just before, and the right-hand picture during, a negative pulse applied to the pipette. A contraction is produced only when the pipette is opposite an I band (image 4). (**c**) Images 5 through 8 are successive frames of a cine film showing the nature of shortening induced by a local depolarization with a 4 **μ**m pipette. The onset of a negative pulse applied to the pipette occurs between images 5 and 6. Note that the contraction spreads inward but not longitudinally (Huxley and Taylor 1958. With permission John Wiley & Sons Inc)

Huxley and Taylor (1955a) concluded that the results were strong evidence that the influence of membrane depolarization was conveyed to the interior of the fiber by spread along some structure in the I band. From the anatomy of the fiber, they believed that "this must almost certainly be Krause's membrane". In his classic review written in early 1955 (Huxley 1957a), Andrew Huxley was somewhat more circumspect in his conclusion when he stated that it was not actually proven that the Z membrane was the structure concerned but rather it may be that the activation was conveyed along some other structure near the middle of each I band. Nonetheless he noted that the Z membrane had the required characteristics and thus it seemed probable

that it was the structure involved in the inward spread of activation. Huxley and Taylor naturally were pleased to have their hypothesis confirmed. But it turned out that there was a problem, a big problem.

Later that year, in November of 1955, Huxley and Taylor presented their results at a meeting of the Physiological Society in London (Huxley and Taylor 1955b). In the audience was J. David Roberston[2] who at the time was working in the department of anatomy at University College London. Huxley (2004) recalled that Robertson "produced from his pocket" a slide showing an electron micrograph of a longitudinal section of muscle which clearly showed a pair of tubules penetrating the fiber on either side of each Z membrane. He therefore suggested that inward conduction took place along these tubules and that the pipettes utilized by Huxley and Taylor were not small enough to distinguish between the two members of each pair. [The slide in question subsequently appeared as figure number ten in a paper published by Robertson (1956).] This suggestion must have been galling to the technical expert Andrew Huxley, not to mention that their hypothesis regarding Krause's membrane would be invalidated. But there was another possibility other than a technical one. Since Robertson's experiment was done on a lizard skeletal muscle and not frog skeletal muscle, there might be a species differences. But given the "uniformity of nature" this possibility seemed somewhat unlikely.

Huxley (1957b) responded to this criticism by doing experiments on crab muscle fibers which have a resting sarcomere length of about 8 μm instead of the 3 μm of the stretched frog fibers. To his surprise, what he found confirmed Robertson's prediction. The sensitive spots along the sarcomere existed at the boundary between the A and I bands and not at the Z line. Only the adjacent half-I band shortened and pulled the Z line towards the A band. Furthermore Huxley confirmed the earlier results from the frog fibers. Subsequently Ralph Straub (1928–1988) joined the Huxley laboratory from Geneva and they (Huxley and Straub 1958) determined that in lizard muscle the sensitive spots appeared at the A/I boundary and not at the Z line just as Robertson predicted. Thus the inward spread could not in general be attributed to Krause's membrane.

Huxley (1957b) made another important observation in frog muscle fibers. The sensitivity did not extend continuously along the Z line but was confined to spots separated by distances of the order of 5 μm around the fiber. With regard to this observation, Huxley and Taylor (1958) concluded that each fiber contained networks

[2] J. (James) David Robertson, M.D., Ph.D. (1923–1995) was a pioneer in the use of the electron microscopy to study cell membrane structure. After receiving his M.D. from Harvard University, Robertson joined F. O. Schmidt's laboratory at MIT in the late 1940s to learn electron microscopy. He received a Ph.D. from MIT in 1952. He then went to the anatomy department at University College London in 1955 and while there Robertson described in 1957 the "unit membrane" hypothesis at a meeting of the Physiological Society to explain the structure of the cell membrane (Robertson 1958). This hypothesis became the fore runner of the "fluid mosaic" model of the cell membrane developed by S. J. Singer and G. L. Nicholson in 1972. Robertson is also credited with the discovery of the structural basis of electrical synaptic transmission. He spent most of his career at Duke University where he was the chair of the anatomy department. For more information, see an autobiographical chapter (Robertson 1987).

of tubules, electrically continuous with the external fluid, in the transverse planes corresponding to the positions at which surface depolarization was effective and that reduction of the potential difference across the walls of these tubules activated the myofibrils. The work on frog and crab muscle fibers subsequently was published in full in 1958 in the same issue of the Journal of Physiology that included the full publication of the Huxley and Niedergerke (1958) sliding filament paper. These two classic papers were published "back to back".

There was no indication that the physiological data was wrong but the picture was confusing. The sensitive spots occurred in different parts of the sarcomere in different muscle species. A no doubt somewhat frustrated Andrew Huxley (1971) noted that this was not what might be expected from phylogenetic relationships. What was needed was a deeper understanding of the internal membrane structure of muscle fibers. It turned out that this understanding also was evolving in the 1950s.

4.4 Sarcoplasmic Reticulum, Triads and Transverse Tubules

In 1953, cell biology pioneers H. Stanley Bennett and Keith R. Porter, using electron microscopy, described an intracellular membrane system in skeletal muscles of the fowl. The membrane system, located between the myofibrils, exhibited longitudinal and transverse components. They named the structure the sarcoplasmic reticulum in analogy to the endoplasmic reticulum observed in other cells. Bennett (1955) suggested that the sarcoplasmic reticulum might be responsible for conveying the inward spread of activation seen by Huxley and Taylor (1958). Bennett (1956) has stated that he and Porter actually rediscovered the sarcoplasmic reticulum with the electron microscope. He held this view because the nineteenth century light microscopists already had described such a system (Bennett 1956, 1960).

The most meticulous study of the reticular apparatus of striated muscle was conducted by Emilio Veratti (1872–1967) at the University of Pavia in 1902. Bennett (1956) believed that this paper was one of the most important ever published on muscle structure. So enthusiastic was Bennett about Veratti's results that he helped translate the paper from Italian and had it re-published in the Journal of Biophysical Biochemical Cytology (Veratti 1961). What did Veratti observe? Using the "black reaction" (precipitation of silver chromate) discovered by Camillo Golgi in 1873 (the Golgi stain) (Mazzarello 2002), Veratti observed in the light microscope transverse and longitudinal structures in fibers of muscles of various types. As impressive as this work was, it was completely forgotten along with the other early studies until the rediscovery of the sarcoplasmic reticulum in the 1950s using electron microscopy. In truth the study was ahead of its time. We will see below that Veratti's results regarding transverse structures in muscle fibers dovetail almost exactly with the location of the sensitive spots seen by Huxley and Taylor in various muscle types.

Porter and George Palade, who would go on to win a Nobel Prize, made a comprehensive study of the sarcoplasmic reticulum with the electron microscope (Porter

and Palade 1957). They examined the sarcoplasmic reticulum of amphibian 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". It was a membrane delineated system. They found that an elaborate lacework or reticulum of tubular and vesicular elements existed as a structural component of the interfibrillar sarcoplasm. This reticular component was continuous in such a way that if the myofibrils were removed, it would appear as a fine "honeycomb". They considered it to be a sleeve around each myofibril.

This sarcoplasmic reticulum was further characterized at the level of the Z line in amphibian muscle. At the Z line the reticulum became dilated. The dilated portion of one sarcomere faced an equivalent dilated portion in the next sarcomere. The dilated portions of the sarcoplasmic reticulum were called terminal cisternae by Porter and Palade (1957). A space of about 500 Å existed between the dilated pairs of the sarcoplasmic reticulum. 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 smaller vesicular units, constituted a three-component structure that was named the triad. Thus one triad existed at the level of each Z line in amphibian muscle.

An important advance was made in 1959 by Ebba Andersson-Cedergren, a graduate student, working in Fritiof S. Sjostrand's laboratory in the department of anatomy at the Karolinska Institute in Stockholm. In an electron microscopic study employing serial sections of mouse muscle fibers, Andersson-Cedergren (1959) noted that one part of the network consisted of transversely oriented tubules which appeared as rows of small vesicles. This part extended from the plasma membrane across the muscle fiber close to the level of the A-I boundary and appeared to form a transversally oriented system, which was not continuous with the rest of the sarcotubular system. She referred to this system as the *transversal or T system*. The middle component of the triad, the T system, consisted of elements which in longitudinal sections usually looked like fragments of tubules or vesicles. However, in cross sections through the muscle fibers the T system consisted of tubules with a very complicated convoluted shape. The three dimensional reconstructions showed that the convoluted tubules appeared to form continuous components extending over long distances across the muscle fiber.

Thus the there is not one intracellular membrane system but rather two discontinuous intracellular membrane systems. One component is the sarcoplasmic reticulum or SR and the other the transverse tubular system or T system. The T system is not made up of a series of small vesicles as suggested by Porter and Palade (1957) but is rather a complicated tubular system running across the muscle fiber. These days, these tubules often are referred to as transverse tubules or T tubules. Finally the triads in the mouse fibers did not exist at the Z line, as in frog fibers, but were observed at the boundary between the A and I bands.

This structural information helped greatly in interpreting the data of Huxley and Taylor (1958). The pieces were starting to come together but unfortunately not all of the pieces. Based on the structural studies, the sensitive spots of Huxley and

Taylor likely represented places where the T system was located in the sarcomere. Thus the T system might convey the surface depolarization into the muscle fiber leading to muscle contraction. In a review in 1960, Bennett emphasized that the transverse structures observed by Veratti in 1902, and rediscovered in the 1950s by electron microscopy, were precisely located where the sensitive spots were observed by Huxley and Taylor in frog and lizard muscle. In fact the transverse tubules were the components that best fitted Veratti's drawings. He didn't actually detect the sarcoplasmic reticulum as defined today (Franzini-Armstrong 2002).

But there was one complication. Lee Peachey, working with Andrew Huxley, observed tubules with the electron microscope in crab fibers at the Z line (Peachey 1959) and later also at the A/I boundary (Peachey and Huxley 1964) but the sensitive spots observed in crab fibers by Huxley (1957b) were only observed at the A/I boundary! The tubules at the A/I boundary were associated with the sarcoplasmic reticulum in the form of a dyad and the tubules at the Z line were not associated with the sarcoplasmic reticulum. It turned out that the presence of the sarcoplasmic reticulum in association with the transverse tubules would make all the difference from the viewpoint of muscle activation (see below).

A major technical advance occurred in 1963 when David D. Sabatini, Klaus Bensch and Russell J. Barrnett then at Yale University introduced glutaraldehyde fixation into electron microscopic studies. Glutaraldehyde is an effective cross-linking agent for proteins and it turned out to be far less disruptive to cell membrane structures than osmium tetroxide fixation. Virtually immediately electron microscopists shifted from osmium tetroxide to glutaraldehyde fixation. One of those microscopists was Peachey (1965) at the University of Pennsylvania who undertook an extensive, quantitative study of structure of the sarcoplasmic reticulum and transverse tubules in frog skeletal muscle. He confirmed the results of Porter and Palade (1957) and Andersson-Cedergren (1959) and added quantitative information. The T system exhibited a volume of 0.3 % of the fiber volume and a surface area about seven times greater than the outer cylindrical surface area of a typical fiber 100 μm in diameter. He produced a beautiful electron micrograph showing the components of the sarcoplasmic reticulum and the transverse tubules (Fig. 4.2) and his schematic illustration of these structures is now considered to be a classic (Fig. 4.3).

4.5 Mechanism of Inward Spread of Activation

Despite the uncooperative crab, the focus now concentrated on the possible role of the T system in muscle activation. Important questions included: did the T tubules open to the muscle surface? Were the T tubules invaginations of the surface membrane or a separate system? Did electrical activity spread into the T tubules by passive electronic spread or a propagated action potential? And, of course, ultimately how does this inward spread of electrical activity lead to muscle contraction?

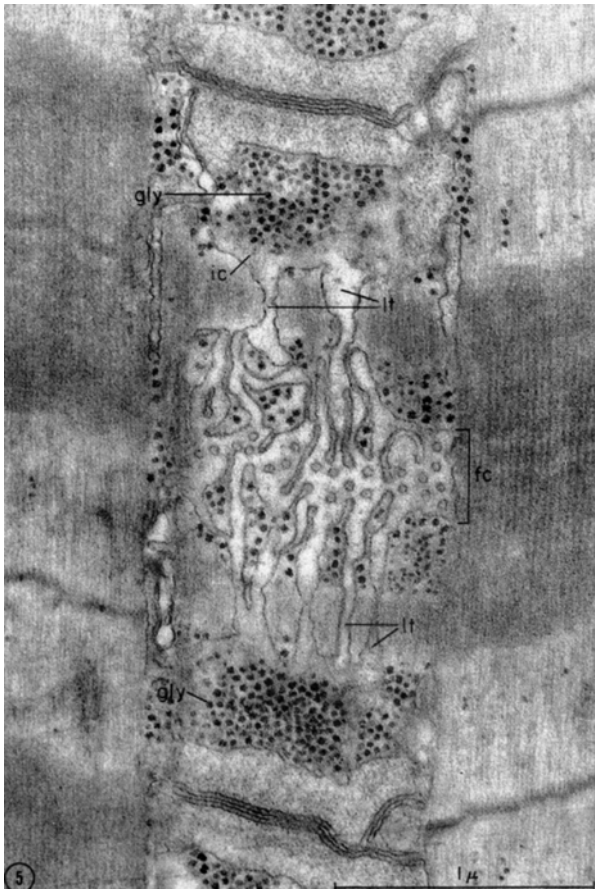

Fig. 4.2 Longitudinal electron micrograph of frog skeletal muscle showing the sarcoplasmic reticulum and transverse tubular system in relation to the myofibrils. In this very thin section, the central vertical strip of the area shown is almost entirely confined to the sarcoplasm between two myofibrils. The underlying myofibril is in register with the myofibril at the right side of the figure. Triads appear at the *top* and *bottom* of the figure, opposite Z lines. Details of the sarcoplasmic reticulum are shown. The section passes through the glycogen masses. Magnification ×60,000 (Peachey 1965. With permission Rockefeller University Press)

4.5.1 Are the Transverse Tubules Open to the Surface?

A flurry of structural and functional studies appeared in 1964 which proved that the transverse tubules were open to the surface and were likely invaginations of the surface membrane. Clara Franzini-Armstrong (Fig. 4.4) and Porter (1964) provided convincing electron microscopic evidence that the transverse tubules were invaginations of the surface membrane in fish striated muscle. They utilized glutaraldehyde fixation to fix muscles of the fish, Black Mollie, a small fish 2–3 cm in length. They did something 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 are beautiful (Fig. 4.5). They concluded that the T system was a sarcolemmal derivative that retained its continuity with the sarcolemma and limited a space that was in direct communication with the extracellular environment. Furthermore they observed about 20–30 transverse tubules at each Z level

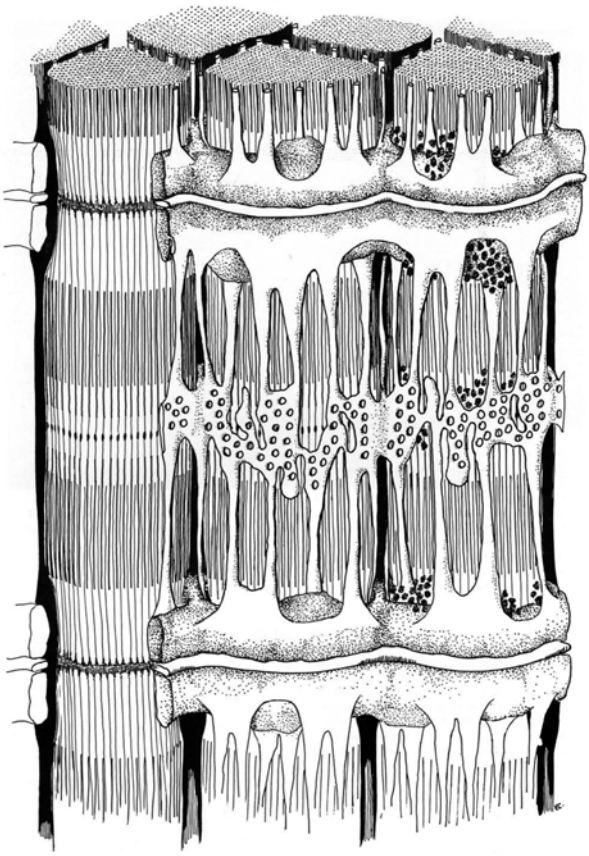

Fig. 4.3 Three-dimensional reconstruction of the sarcoplasmic reticulum and transverse tubular systems associated with several myofibrils in the frog. Magnification approximately ×40,000 (Peachey 1965. With permission Rockefeller University Press)

around the circumference of the small 12 μm diameter fish fibers. This result is reminiscent of the circumferential separation of sensitive spots at the Z line in frog fibers (Huxley 1957b). Further evidence that the transverse tubules were open to the surface came from the observations by Makoto Endo[3] (1964) in Andrew Huxley's laboratory. When a frog fiber is soaked in a solution containing a charged fluorescent dye that does not penetrate the muscle cell membrane and the solution then removed, the fiber exhibits striations in the fluorescence microscope. Upon rapidly shifting to a polarizing microscope it was observed that fluorescent striations

[3] Makoto Endo came to Andrew Huxley's laboratory in the autumn of 1962 from Hiroshi Kumagai's laboratory at the University of Tokyo. In Japan he learned biochemistry and physiology from Setsuro Ebashi. After his stay in the Huxley laboratory he returned to Japan and spent most of his distinguished research career at the University of Tokyo. He is best known for his work describing calcium-induced calcium release in muscle (Endo et al. 1970), a mechanism that is prominent in the heart. In 1957 Professor Kumagai organized the first international conference on the chemistry of muscular contraction to be held in Japan after World War II.

Fig. 4.4 Clara Franzini-Armstrong (b. 1938) (*right*): Her electron microscopic studies on the transverse tubular system and its relationship to the sarcoplasmic reticulum span more than 40 years and are considered foundational and pioneering in muscle field. She received her degree (Laurea) from the University of Pisa in 1960. She did postdoctoral training with Keith Porter at Harvard University, worked with Andrew Huxley at University College London and Richard Podolsky at the NIH. She has spent the majority of her career at the University of Pennsylvania. She was elected to the National Academy of Sciences in 1995 and as a foreign member of the Royal Society London in 2001. She received the Founders Award from the Biophysical Society. She became professor emerita in 2007 at the University of Pennsylvania. Her husband Clay Armstrong (*left*), the eminent electrophysiologist, also is a member of the National Academy of Sciences. Photo: courtesy C. Franzini-Armstrong

occurred in the middle of the I bands. The fluorescence then quickly faded suggesting that the dye was washed out of the transverse tubules without entering the main part of the fiber. Rather than a dye, Huxley (1964) and independently Page (1964) showed that ferritin could enter the transverse tubules of frog skeletal muscle. Ferritin consists of a protein shell enclosing a dense core of ferric hydroxide with each ferritin containing about 3,000 iron atoms. The electron dense ferritin was easy to detect in the electron microscope. The results (Huxley 1964) clearly showed that ferritin could enter the transverse tubules (Fig. 4.6). Huxley (1964) concluded that the transverse tubular system opened directly into extracellular space and that ferritin entered by free diffusion. He also concluded that there was continuity of the transverse system across the fiber diameter. A dramatic affirmation of this conclusion is seen in the scanning electron micrographs (Fig. 4.7) of the surface of a frog muscle fiber by McCallister and Hadek (1970). Openings on the surface occur at the Z line and circumferentially around the fiber just as predicted by the electrophysiological experiments of Huxley and Taylor. There was no disagreement now, the transverse tubules were invaginations of the surface membrane.

These experiments also re-enforced the conclusion that the T system is separate from the sarcoplasmic reticulum. The T system is an invagination of the surface membrane whereas the sarcoplasmic reticulum exists totally within the fiber. Certainly they are in close apposition at the triad but is there any connection between

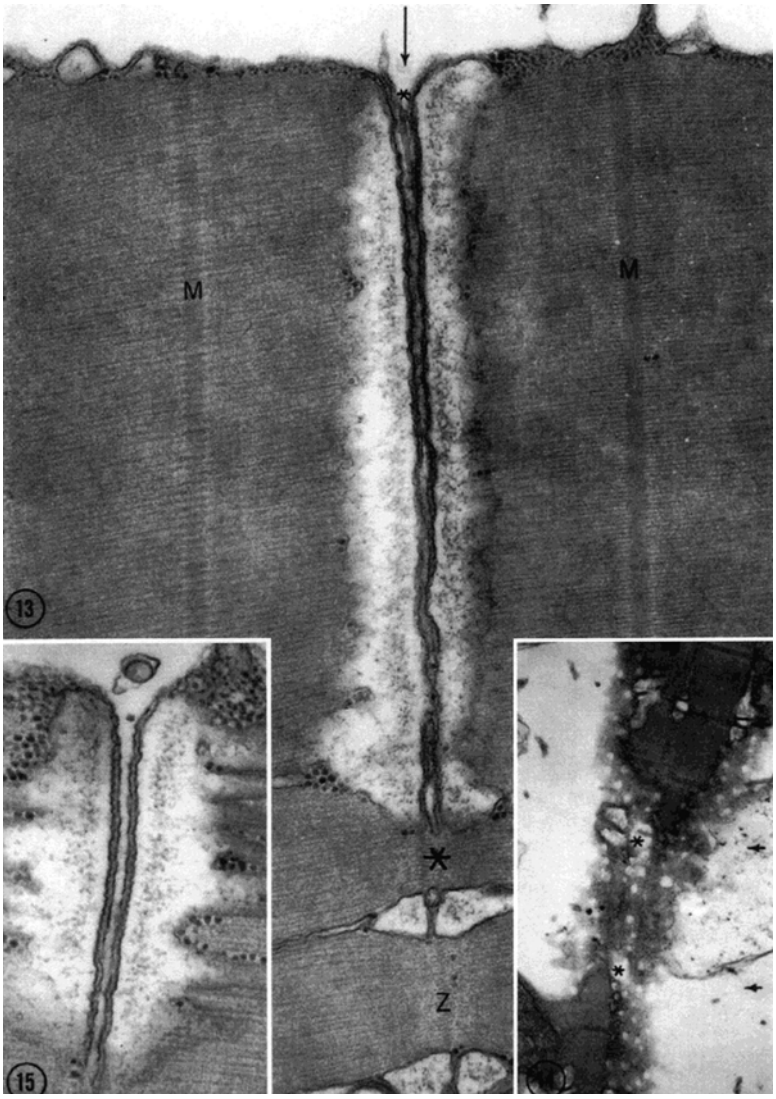

Fig. 4.5 Longitudinal electron micrographs of the T system in fish muscle fibers. These images provide a lateral view of the T system and terminal cisternae of the sarcoplasmic reticulum. Note that the T system is located at the Z line, opens to the surface and exhibits a close relationship with the sarcoplasmic reticulum. Magnification ×46,000–×60,000 (Franzini-Armstrong and Porter 1964. With permission Rockefeller University Press)

the two systems? Franzini-Armstrong (1970) showed that there were structures that reached across the 100–150 Å gap from the sarcoplasmic reticulum to the T system. At periodic intervals of about 300 Å the sarcoplasmic reticulum membrane forms small projections whose tips are joined to the T system membrane by some amorphous

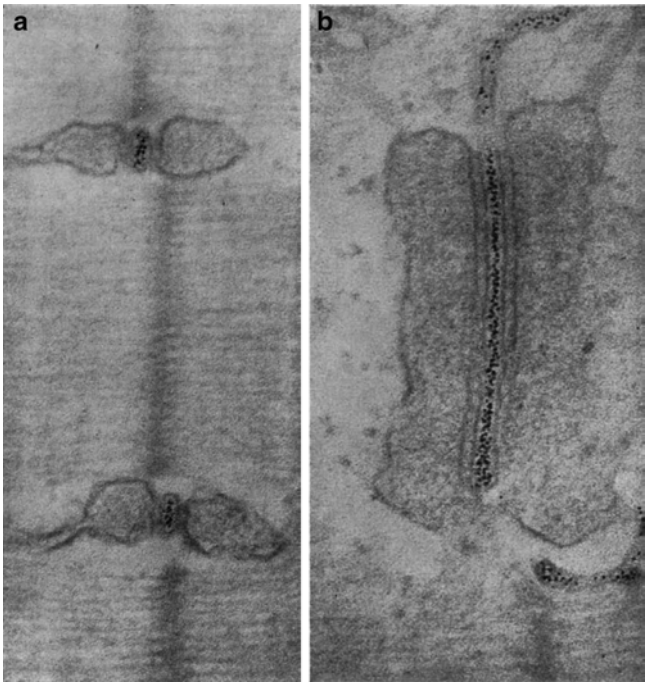

Fig. 4.6 Electron microscopic views of triads in ferritin treated frog muscle fibers. (**a**) A section perpendicular to transverse tubules filled with ferritin molecules. (**b**) A section parallel to a transverse tubule indicating penetration of ferritin molecules throughout the tubule. Magnification ×145,000 (Huxley 1964. With permission Nature Publishing Group)

material (Fig. 4.8). She called these processes "SR feet". Over time these foot processes sometimes whimsically have been called "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 coupling process (see Chap. 8).

Various other experiments could now be given a ready explanation. The observation by Bernard Katz (1948) of a fivefold higher capacitance in skeletal muscle fibers than in nerve fibers could now be attributed to the extra membrane area of the T system. Hodgkin and Horowicz (1960) made an extensive study of the effects of K^+ solutions on membrane potential and contraction in frog muscle fibers. When a single muscle fiber was exposed to a solution of elevated K^+ concentration, the membrane depolarized to a steady level. When that solution was rapidly removed, the membrane repolarized but at a much slower rate than the rate of depolarization. Hodgkin and Horowicz (1960) speculated that the K^+ was retained for a short time in "a special region" in the fiber. This region was most likely the T system of the fiber from which K^+ that entered would only slowly diffuse out. Freygang et al. (1964) studied the effects of repeated stimulation on the shape of the action potential of frog muscle fibers. Skeletal muscle action potentials exhibit a tail or negative

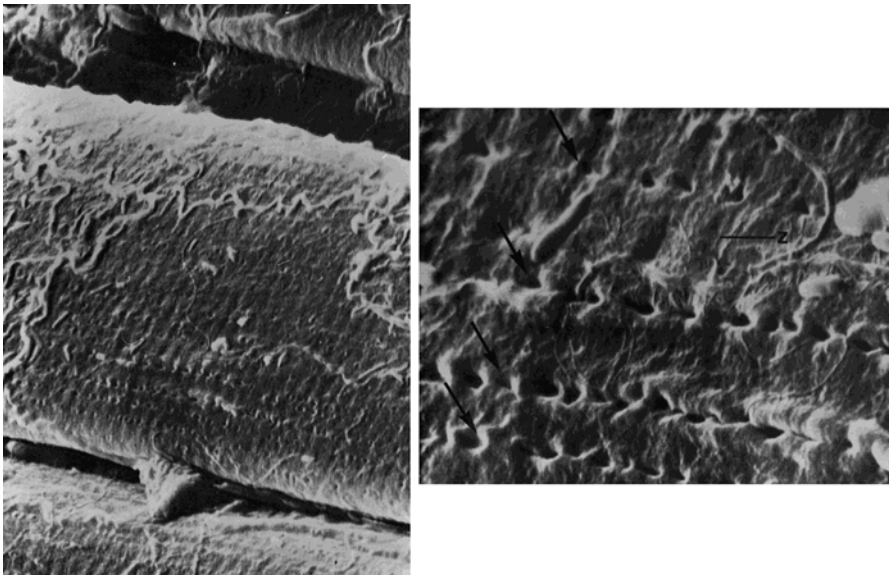

Fig. 4.7 Scanning electron micrographs of the surface of a frog muscle fiber. *Left*: lower magnification (×2,500) showing the striated pattern of the myofibrils that is evident at the fiber surface with the lighter ridges corresponding to the A bands and the dark furrows corresponding to the I bands. *Right*: higher magnification (×10,000) where *arrows* indicate the parallel rows of openings corresponding to the T system. The openings are found at the level of the I band in the vicinity of the Z line and are distributed circumferentially around the fiber (McCallister and Hadek 1970. With permission Elsevier)

after-potential. With repeated stimulation this after-potential became larger and returned to the resting level more slowly than after a single stimulus. They interpreted this observation as the accumulation of K^+ during the repeated stimulation "in an intermediary space" that was located between the major portion of the sarcoplasm and the external fluid. All of these experiments suggested that the T system is in electrical continuity with the surface membrane.

The conclusion that the unique electrical properties of skeletal muscle fibers could be attributed to the presence of the T system soon was confirmed in dramatic fashion. John N. Howell (Howell and Jenden 1967; Howell 1969), a graduate student at UCLA at the time, made the seminal observation. If a frog muscle is soaked in a solution made hypertonic by adding glycerol, the transverse tubules became disrupted when the muscle was returned to an isotonic salt solution. These muscles were no longer responsive to stimulation. Brenda R. Eisenberg (now Brenda Russell) and Robert (Bob) S. Eisenberg (1968) showed that this treatment destroyed 98 % of the transverse tubules. Bob Eisenberg and Australian physiologist Peter W. Gage (1967) made an extensive study of the electrical properties of these muscle fibers. They found that action potentials did not produce a mechanical response and that the negative after-potential was absent after a single stimulus or a train of stimuli.

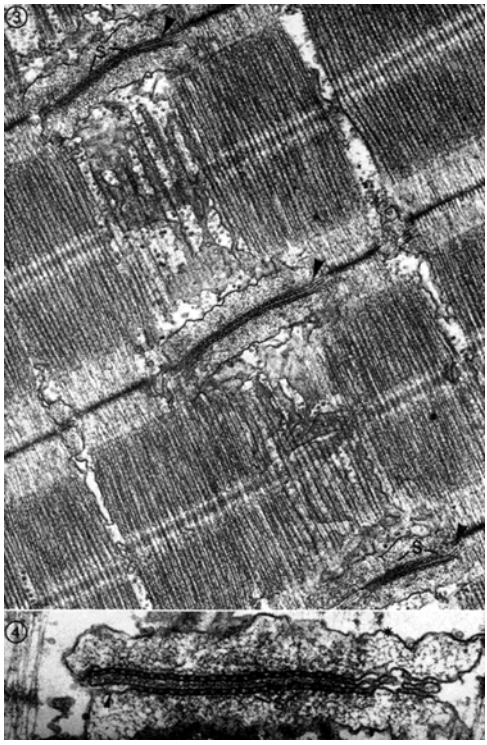

Fig. 4.8 Electron micrographs showing periodic structures connecting the T system to the sarcoplasmic reticulum in frog muscle fibers. These structures have been called "SR feet" by Franzini-Armstrong. Magnification ×45,000 above and ×100,000 below (Franzini-Armstrong 1970. With permission Rockefeller University Press)

Also the membrane capacitance was strikingly reduced. Thus the matter was settled: the T system was in electrical contact with the surface membrane, responsible for the characteristic properties of the muscle action potential and necessary for muscle activation.

4.5.2 Is the Spread of Activation into the T System Active or Passive?

Huxley and Taylor (1958) interpreted their results to indicate that inward spread of electrical activity into the muscle fiber was a passive process. They based this interpretation upon the observation that the distance to which the contraction spread inwards from the fiber surface was graded according to the strength of the pulse applied. Nonetheless, they still considered the mechanism of inward spread to be an open question. Hugo Gonzales-Serratos (d. 2011) came to Andrew Huxley's laboratory from Mexico in the early 1960s to work for a Ph.D. He devised a remarkable experiment to determine the velocity of inward spread of contraction in a single frog muscle fiber. Gonzalez-Serratos compressed a frog fiber in gelatin until all the

myofibrils became wavy. When the fiber was stimulated to produce a twitch, the active myofibrils straightened and this spread of inward contraction was recorded with high-speed cine microphotography at a remarkable 2,000–3,000 frames per second (Gonzalez-Serratos 1966, 1971). The contraction spread inward with a propagation velocity of 7 cm/s at 20 °C. Thus it would take less that 1 ms for the contraction to reach the middle myofibrils of a typical 100 μm frog fiber. As the temperature was decreased the propagation velocity decreased with a Q_{10} of about 2. Passive electronic spread would be expected to be almost temperature insensitive. Thus these results suggested for the first time that the propagation of the excitatory process along the tubules was probably a regenerative process like an action potential rather than a passive one. When Andrew Huxley gave his Croonian lecture in 1967 (Huxley 1971), he still wasn't completely convinced but did finally acquiesce when the results of LeRoy (Roy) L. Costantin (1970) clearly indicated that a Na^+ current in the T tubule membrane contributed to the tubule spread of the excitatory process, as in an action potential. The radial spread of contraction was less effective in fibers exposed to a bathing medium with greatly reduced Na^+ concentration or to tetrodotoxin which was known to block the increase in Na^+ permeability associated with an action potential. The results strongly supported the existence of a sodium-regenerative process in the T system. For one of the few times in his research career Andrew Huxley got it wrong. The inward spread of electrical activity leading to muscle contraction was active and not passive.

Thus the first steps of excitation-contraction coupling as defined by Alexander Sandow were elucidated both functionally and structurally.[4] What was missing in 1958 was a general understanding of what happened next. Huxley and Taylor (1958) emphasized that there was little evidence concerning the final step from the hypothetical transverse networks to the filaments themselves. It turned out that there was evidence that Ca^{2+} played a crucial role in muscle activation but much of this evidence was either forgotten or ignored.

4.6 Ca^{2+} and Contractility: The Early Studies on Muscles

The history of Ca^{2+} and skeletal muscle contraction is a curious one. It involved forgotten clues, feuds around the merits of doing experiments on "living" muscles versus "dead" muscle components and strong personalities. There were different "clubs", the cell physiologists and the biochemists, who spoke different "languages" and who weren't able or willing to consider the contributions of the other club. For the biochemist, there were many confounding issues (Franzini-Armstrong 1998).

[4] Jozef Zachar (1925–2000) produced in 1971 a remarkable monograph entitled: "Electrogenesis and Contractility in Skeletal Muscle Cells" which covers excitation-contraction coupling in great experimental detail, especially from a comparative muscle point of view. At the time of the publication, he was a Professor at the Institute of Normal and Pathological Physiology, Bratislava, Czechoslovakia (now the Slovak Republic).

Some of these issues included: variable and unknown Ca^{2+} contamination of reagents, especially ATP; contamination due to Ca^{2+} leaching from the glassware; variable purity of protein preparations; and the difficulty of detecting the low concentrations of Ca^{2+} that produced contraction of the model systems. Also there was the confusing issue that ATP could cause contraction of a muscle model system but also relaxation. Finally there was the problem that many biochemists had difficulty in believing that something as simple and common as the Ca^{2+} could regulate something as important as human movement. In the end, the Ca^{2+} hypothesis of contraction would become accepted but not without much hard work and some famous experiments.

The cell physiologists found the early clues which for reasons described below were set aside. They believed strongly that the best, maybe only, way to gain a full understanding of cellular function was to study living cells. Books with titles such as "The Dynamics of Living Protoplasm" by Heilbrunn (1956) and "Explorations into the Nature of the Living Cell" by Robert Chambers and his son Edward L. Chambers (1961) clearly expressed the authors' point of view. They took a general physiology approach and looked for commonality in cell function across the animal kingdom. In a study published in 1932, Robert Chambers and H. P. Hale examined the effects of freezing and thawing on different cells including segments cut from frog muscle fibers. They routinely used the response to Ca^{2+} as an indicator of a healthy fiber. The contractility of the fibers was tested by blowing a small quantity of 0.1 M $CaCl_2$ with a micropipette on the surface of the fiber. They noted that this procedure induced a pronounced contraction of a healthy fiber. (The Ca^{2+} likely entered the fiber segment through the cut ends.)

In a paper entitled "the action of calcium on muscle protoplasm" in 1940, Lewis Victor Heilbrunn[5] at the University of Pennsylvania described in detail his Ca^{2+} release theory of muscle contraction. He proposed that in a muscle fiber at rest, Ca^{2+} was stored in the cortex of the cell, a 10 μm thick region just under the cell membrane. This Ca^{2+} was then released into the more or less fluid portion of the cell interior to cause contraction upon muscle stimulation. In support of his hypothesis, he observed that when cut frog fibers were exposed to an isosmotic solution of $CaCl_2$ (90 mM) there was a strong contraction and the fiber segment lengths decreased by 70 % (Fig. 4.9). He believed that the Ca^{2+} diffused into the fiber segment through the cut ends.

[5] Lewis Victor Heilbrunn (1892–1959) spent the majority of his career in the department of zoology at the University of Pennsylvania where he trained more than 50 scientists for the Ph.D. He was a prophetic author who wrote his first book, The Colloid Chemistry of Protoplasm, in 1928. He was a true general physiologist whose research interests ranged from the study of protoplasm in cell division, ameboid movement, fertilization and muscle contraction. He was a strong personality, a decorated aviator in World War I, who held steadfastly to his scientific beliefs. He is best known for his advocacy for the universality of the role of Ca^{2+} in regulation of cell function. Unfortunately he did not live long enough to see his idea vindicated. He died in an automobile accident in 1959. He would have gotten great joy out of Anthony K. Campbell's book entitled: Intracellular Calcium—Its Universal Role as Regulator (1983). For more on Heilbrunn, see Steinbach (1960), Shreeve (1983) and Gross (1986).

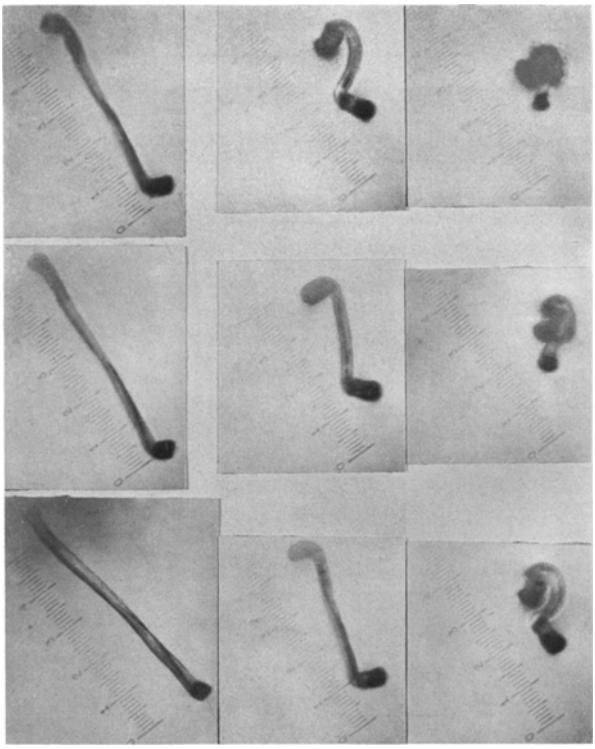

Fig. 4.9 Shortening of an isolated muscle fiber in isosmotic $CaCl_2$. The fiber was cut from a frog muscle. The succession of stages is from *left* to *right*. The first photograph was taken in Ringer's solution; the second, 10 s after addition of $CaCl_2$. Subsequent pictures were taken at 10 s intervals (Heilbrunn 1940. With permission The University of Chicago Press)

Kamada and Kinosita (1943) of the Tokyo Imperial University confirmed Heilbrunn's observations in a paper extraordinary for its time, its place and elegance of techniques. But the paper was virtually unknown in the west until well after World War II. They investigated the effects of various solutions on the contraction or in their words "coagulum formation" of isolated, intact or cut, frog muscle fibers. They injected solutions into the fibers through micro-pipettes with tip diameters of 2–5 μm. Most effective in producing contraction of the naturally occurring ions was a solution containing 83 mM $CaCl_2$. If the amount of injected solution was small, the local contraction was followed by relaxation.

Heilbrunn and Wiercinski (1947) conducted a study that was at first considered influential, then set aside and now considered classic. Their point of view was made clear when they emphasized that the phenomenon of muscular contraction occurs in living rather than in dead muscle. They also utilized single cut fibers from the frog. Ironically, many physiologists would consider these cut fibers to be "dead" or at least injured. These cut fiber segments were immersed in a Ca^{2+} free solution. Various solutions were injected into the fiber segments through a micropipette and the extent of segment shortening was measured. Of the physiologically occurring ions only Ca^{2+} resulted in a strong contraction with an average fiber segment shortening of 44 % in a few seconds. Solutions with a Ca^{2+} concentration as low as

200 μM produced a contraction. Considering that the amount of solution injected into the fiber segment was about 10 % of the segment volume, this suggests that the steady Ca^{2+} concentration might have been as low as 20 μM. The fiber segments did not relax. Heilbrunn believed that muscle contraction was a process analogous to blood clotting. In contrast, injection of solutions resulting in a final fiber segment concentration of about 8 mM $MgCl_2$ or 12.6 mM KCl or 12.3 mM NaCl produced only small changes of the fiber segment length (less than 10 %). The results were detailed and convincing. This paper and the accompanying hypothesis of Ca^{2+} regulation of contraction at first were influential but only for a very short time.

There was a problem with Heilbrunn's Ca^{2+} theory of muscle contraction, a fatal problem. In 1949 A. V. Hill showed that it was impossible to explain the rapidity of frog muscle contraction by diffusion of a substance from the cortex of the cell (see above). Hill was a Nobel Laureate and his word was "instant law" (Shreeve 1983). Thus Heilbrunn's hypothesis was set aside. In a sense the "baby (Ca^{2+}) was thrown out with the bath water" (location of Ca^{2+} in cell cortex at rest). Of course these events occurred before the recognition of the inward spread of electrical activity in a muscle fiber.

There was another reason that Heilbrunn's work was discredited and that was that Heilbrunn's myopic view of the work of others, especially the biochemists, made matters worse. He believed that Ca^{2+} was a universal regulator of cellular function in all cells. His perceived over insistence on the role of Ca^{2+} as a universal regulator led some of his colleagues to call him a "calcium maniac" (Shreeve 1983). That he was a first rate scholar there could be no doubt. Besides his original publications, he also published single authored books. Examples include his textbook entitled "An Outline of General Physiology" that went through three editions and his monograph the "Dynamics of Living Protoplasm". In an unusually candid assessment of Heilbrunn some years after his death, Gross (1986), then director of the Marine Biological Laboratory at Woods Hole where Heilbrunn spent many summers, characterized Heilbrunn as a remarkable scholar. But he went on to state that Heilbrunn was committed to an outmoded way of thinking about cell chemistry and structure. He didn't believe that the function of a cell could be understood by looking at its individual pieces. He was dogmatic and disdainful of the biochemists whose influence was ascending dramatically in the 1940s and 1950s. For example, concerning the then confusing dual role for ATP in muscle model systems, Heilbrunn (1956) had this to say (Heilbrunn 1956. With permission Elsevier):

> One thing is certain, adenosine triphosphate, or ATP, is a powerful word in all our modern thinking about muscle…words seem to have taken the place of the incantations used by magicians in the days before modern science. Whereas the old magicians were content with their abracadabra, we now have much more impressive words like adenosine triphosphate. Adenosine triphosphate—and the muscle contracts; adenosine triphosphate and it relaxes again.

And there was more, Heilbrunn believed that the effect of ATP on dead glycerinated muscle fibers could scarcely be the same as that on the living fibers. Ebashi (1980) noted that the fact that Heilbrunn did not seem to appreciate the role of ATP may have discredited the concept that Ca^{2+} itself was important at the molecular level. So it is not surprising that Heilbrunn's work garnered little respect from the biochemists. The fact that Heilbrunn spent his summers at the Marine Biological

Laboratory at Woods Hole, Massachusetts, where Albert Szent-Gyorgyi and his group had a year round residence must have led to some tense moments between these two great but dogmatic scholars. Nonetheless, Hill's (1949) criticism came and "abracadabra": Heilbrunn's Ca^{2+} release hypothesis was discarded.

In a somewhat sobering close to his assessment of Heilbrunn, Gross concluded with some advice for young investigators (Gross 1986. No objection from Marine Biological Laboratory. Author could not be located):

> Perhaps they will consider, however, these few generalizations, which *I know* are true: Being right isn't enough. What you say, however right, must be said in a currently acceptable language, must not violate too brutally current taste, and must somehow signal your membership in a respectable professional club. If you want to succeed you must play *all* of the game, not just the innings in laboratory or library.

That wasn't the end of the Ca^{2+} story from a physiological perspective. Niedergerke (1955), after finishing his contribution to the experiments leading to the sliding filament model of muscle contraction with Andrew Huxley, essentially confirmed the observations of Heilbrunn and Wiercinski. He injected Ca^{2+} from a micropipette by electrolytic transport into a living muscle fiber from the frog. The myofibrils near the pipette tip rapidly shortened and slowly lengthened, but not completely, once the current was turned off. Injection of a solution of KCl or $MgCl_2$ did not induce a contraction. Niedergerke concluded that the local contraction and partial relaxation suggested that Ca^{2+} activated a specific link of the contractile cycle. His observations were described in a brief publication in the proceedings of the Physiological Society. Niedergerke was a member of the right "professional club" but unfortunately he did not follow-up these initial observations with a full study. He moved on immediately to study the role of Ca^{2+} in cardiac muscle contraction.

4.7 Ca^{2+} and Contractility: Observations on Model Systems

The early physiological studies provided definite clues for an important role for Ca^{2+} in contraction but these studies were essentially descriptive. It was through detailed biochemical studies on muscle model systems that a molecular picture for the role of Ca^{2+} in contraction emerged. Despite these clues of the role of Ca^{2+} in muscle contraction, Ebashi and Endo (1968) have stated that "It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation." Various simplified model systems were developed to mimic contraction. Some of these model systems included (Ebashi and Endo 1968; also see Chap. 1): (1) reconstituted or "synthetic" actomyosin made from purified actin and myosin, (2) "natural" actomyosin which likely contained other structural proteins made by isolating actomyosin from muscle, (3) actomyosin threads made from synthetic or natural actomyosin, (4) myofibrils, and (5) glycerinated muscle fibers. These systems could be made to "contract" by addition of ATP and Mg^{2+} at an appropriate ionic strength. Contraction of model systems (1), (2) and (4) is represented by superprecipitation and increases in actomyosin ATPase activity. In the presence of Mg.ATP, the actomyosin threads shorten and the

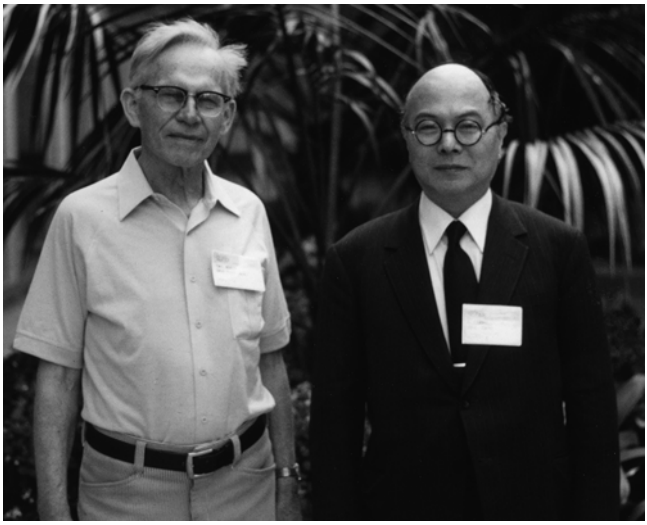

Fig. 4.10 Emil Bozler and Setsuro Ebashi in 1989 at Ohio State University during a symposium on smooth muscle in honor of Bozler. Ebashi's development of the calcium concept was stimulated by the observations of Bozler in 1954 (see Fig. 4.11 and associated text). They were long time friends (Photo: author's collection)

glycerinated fibers develop force and can do work by shortening. The glycerinated fiber preparation is most suitable for mechanical studies. In the view of the biochemists, all of these systems mimic muscle contraction but in none of them was relaxation observed before 1950.

The first important step was taken by Emil Bozler[6] (Fig. 4.10) at Ohio State University. He was interested in the mechanism of skeletal muscle relaxation. He utilized bundles of glycerol-extracted psoas muscle fibers from the rabbit to study relaxation. What he discovered was pathfinding. A bundle of fibers developing a steady force due to the addition of Mg.ATP completely relaxed when exposed to a solution containing the Ca^{2+} chelator ethylenediamine tetraacetate (EDTA) (Fig. 4.11) (Bozler 1954). In turn the bundle could be made to contract once again by addition of a 2 mM $CaCl_2$. Thus Ca^{2+} caused a contraction and Ca^{2+} binding led to relaxation. The interpretation seemed simple but Bozler interpreted his results in a complex way, and as it turned out, incorrect way. He proposed that Ca^{2+} was permanently bound to actomyosin and that the chelator blocked this Ca^{2+} and inhibited contraction. The excess added Ca^{2+} bound to the chelator and contraction ensued. Nonetheless these were

[6] Emil Bozler (1901–1995). born in Steingebronn, Germany, came to the department of physiology at Ohio State University in 1936 and spent his entire career there, becoming a professor emeritus in 1971 and thereafter continuing to work for another 20 years. He is best known for his contributions to smooth muscle physiology where he has been called the "father of smooth muscle physiology". He classified smooth muscles into two categories: unitary (visceral) and multiple-unit. He also discovered the pacemaker prepotential in cardiac muscle using extracellular electrodes in 1943. His publications span more than 60 years from 1925 to 1990. His work was deeply respected in Japan and he and Setusro Ebashi were friends. See Rall (1990).

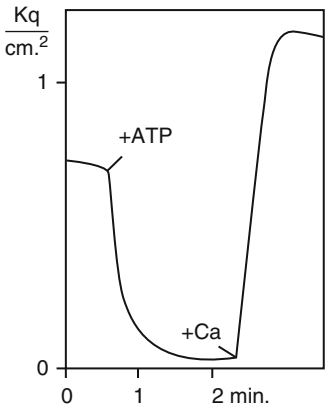

Fig. 4.11 Relaxation of muscle fibers in response to a Ca^{2+} chelator. Skeletal muscle fibers from the rabbit were extracted in a glycerol solution to remove membranes and soluble proteins. Contraction of the fiber bundle was induced by a solution containing ATP and $MgCl_2$. Once steady force was reached, the fiber bundle was exposed to a solution containing the Ca^{2+} chelator EDTA and relaxation ensued. The bundle contracted once again on exposure to the solution containing 1 mM $CaCl_2$ (Bozler 1954. With permission Rockefeller University Press)

Fig. 4.12 Annemarie Weber (1923–2012), M.D., in 1959 was the first to show that Ca^{2+} in the micromolar range regulated the ATPase activity of isolated myofibrils, a model system for skeletal muscle contraction (Franzini-Armstrong 1998. With permission Elsevier)

crucial experiments because for the first time they demonstrated the importance of Ca^{2+} in contraction of a muscle model system and also because they introduced the concept of controlling Ca^{2+} concentration with a Ca^{2+} chelator.

Annemarie Weber (Fig. 4.12) was stimulated by Bozler experiments and in 1959, using EDTA, she was the first to show that isolated myofibrils must be in equilibrium

with a very low concentration of Ca^{2+} in order to have a maximal rate of ATP hydrolysis (Weber 1959). It was difficult to know exactly what that Ca^{2+} concentration was but her data indicated that it was likely to be less than 10 μM free Ca^{2+} concentration. Thus the concentration of Ca^{2+} necessary for contraction in muscles or model systems seemed to be getting strikingly smaller as techniques advanced. It would get even smaller.

At about the same time Setsuro Ebashi[7] (Fig. 4.10) at the University of Tokyo was pursuing the mechanism of muscle relaxation also using model systems. Based on Bozler's results, he reasoned that the extent of relaxation of a glycerinated muscle preparation should vary in proportion to the affinity of the chelator for Ca^{2+}. He obtained six Ca^{2+} chelators with varying affinities for Ca^{2+} to test this idea (Ebashi et al. 1960). They measured the "relaxation activity" as the time that it took to initiate lengthening of a contracted glycerinated fiber bundle after the introduction of the chelator solution. As hoped the chelator with the highest Ca^{2+} affinity, GEDTA (now known as EGTA, ethylene glycol tetraacetic acid), exhibited the greatest relaxation activity, i.e., shortest time to start relaxation. But when all six chelators were considered, there was no correlation between Ca^{2+} affinity and relaxation activity. This result was a great disappointment to Ebashi because it did not support his belief in the role of Ca^{2+} in contraction and relaxation. In 1959 he went to the Rockefeller Institute in New York to work with Fitz Lipmann. Sometime after the June 1959 submission of the paper to the Journal of Biochemistry and before May 1960, Ebashi realized that he made a simple but major mistake. He did not make allowance for the fact that the various Ca^{2+} chelators also had various affinities for Mg^{2+} which he had in his solutions. When this correction was made, the correlation became excellent (Fig. 4.13). In May of 1960 he sent off a letter to the editor of the Journal of Biochemistry and the new result was published in July 1960 (Ebashi 1960). Ebashi (1994) always deeply regretted this error. In the letter to the Journal of Biochemistry (Ebashi 1960), he acknowledged Annemarie Weber's work when he indicated that her work and that of his laboratory indicated the role of Ca^{2+} in contraction of the actomyosin system. So Weber and Ebashi were both thinking along the same lines with regard to role of Ca^{2+} in muscle contraction and relaxation. But there was much more hard work to be done.

One of the main problems was that their fellow biochemists had a difficult time accepting the proposal that something as simple and common as Ca^{2+} could control muscle contraction. There was another problem. The experiments indicating the role of Ca^{2+} in contraction were done in the presence of synthetic calcium chelators

[7] Setsuro Ebashi (1922–2006) was born in Tokyo and received his M.D. degree in 1944 from the University of Tokyo and served as a naval surgeon and was detained in Shanghai after the war. After returning to Japan in 1946, he received his Ph.D. from the University of Tokyo under the mentorship of Professor Hiroshi Kumagai in 1954. He spent the majority of his career in the department of pharmacology at the University of Tokyo. Ebashi received many awards, nationally and internationally, including the Order of Cultural Merit, the highest scientific honor in Japan. He was a member of the Japan Academy and a Foreign Member of the Royal Society and Foreign Associate of the National Academy of Sciences in the United States. For further details see Endo (2006) and Otsuka (2007).

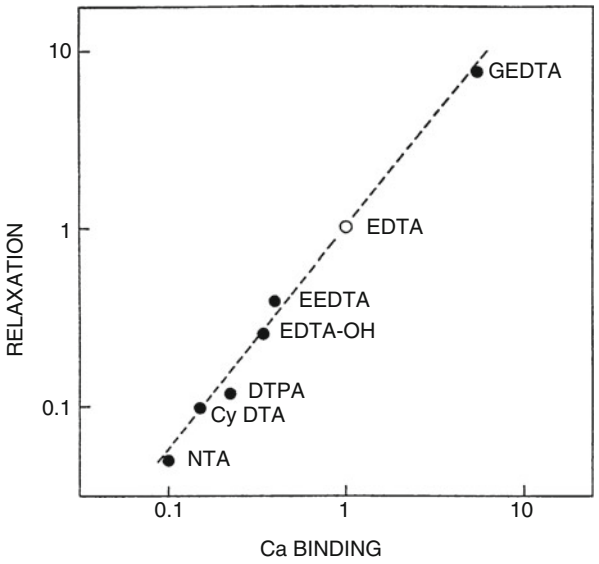

Fig. 4.13 Relationship between calcium binding by various chelating compounds and relaxation of glycerol extracted muscle fibers. Relaxation was measured as the time it took to initiate lengthening of a contracted fiber bundle after the introduction of the chelator solution. Activity of each compound was expressed as its value relative to that of EDTA. Calcium binding was adjusted for the variable binding of Mg^{2+} by the chelators. GEDTA is now referred to as EGTA. For full names of the chelators, see Ebashi et al. (1960) (Ebashi 1960. With permission Oxford University Press)

which, of course, did not occur naturally in muscles. It was important to show the effects of Ca^{2+} in the absence of Ca^{2+} chelators. Given the ease of contamination of reagents and actomyosin with Ca^{2+} in the micromolar range, this would be a heroic experiment. In 1961, Ebashi undertook what he has called the most difficult experiments of his career. He went to extreme lengths to minimize Ca^{2+} contamination in the glassware, reagents and actomyosin preparations before starting the experiment. He isolated "natural" actomyosin from rabbit muscle and measured contraction as the increase in turbidity that accompanied the superprecipitation of actomyosin upon addition of ATP. The time course of superprecipitation was markedly enhanced by Ca^{2+} in the submicromolar range (Fig. 4.14) (Ebashi 1961a). This result was crucial in that it indicated that the effects of Ca^{2+} were not an artifact of Ca^{2+} chelators.

At about the same time, Weber and Winicur (1961) examined the effect of Ca^{2+} on the superprecipitation of a "synthetic" or reconstituted actomyosin system, i.e., purified myosin and purified actin. This system, of course, was the most basic model of muscle contraction. The superprecipitation and accompanying ATPase activity was generally inhibited in the presence of EGTA, but not always. This was potentially a major problem. Some of their preparations were Ca^{2+} insensitive. In their summary, they stated that some actomyosin preparations were inhibited only to a small extent by EGTA and that this effect was a property of the actin moiety. Thus

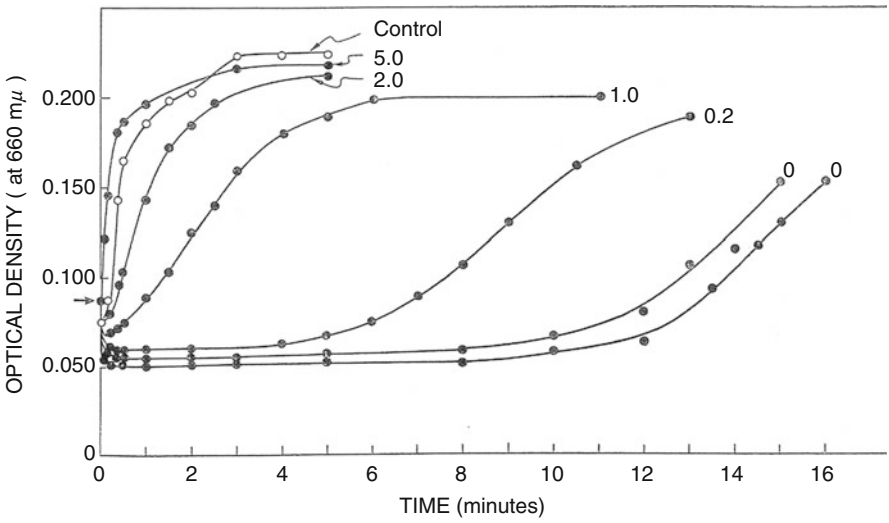

Fig. 4.14 Superprecipitation of natural actomyosin as a function of time in the presence of variable concentrations of free Ca^{2+}. Superprecipitation was measured by following the increase in turbidity. This increase in turbidity was taken as a measure of contraction. Ca^{2+} concentration ranged from 0 added to 5 **μ**M. One of the two tests containing no added Ca^{2+} was done at the beginning of the series of this experiment and the other, at the end. Note there is a substantial acceleration of superprecipitation of actomyosin with a Ca^{2+} concentration as low at 2 **μ**M (Ebashi 1961a. With permission Oxford University Press)

not all synthetic actomyosin preparations were the same. Some preparations were different and this difference may somehow be associated with actin itself. Earlier Perry and Grey (1956), in a short note, also observed that the ATPase activity of synthetic actomyosin was insensitive to EDTA addition. Weber and Herz (1963) went on to show that exchangeable Ca^{2+} bound to myofibrils in the submicromolar range and enhanced, in a complex relationship, superprecipitation and ATPase activity (Fig. 4.15). Thus the free Ca^{2+} concentration necessary to cause contraction in these model systems was remarkably low. Weber and Herz also noted that there were "nonrelaxing" actomyosins which superprecipitate even in the presence of a Ca^{2+} chelator. These experiments were especially complex with very low Ca^{2+} concentrations necessary, possible Ca^{2+} contamination from various sources, multiple agents that bind Ca^{2+} and Mg^{2+} and even uncertainly about the protein nature of the preparations.

Despite the barriers, Weber and Ebashi made much progress but still not all the pieces fit together and people were not convinced. Ebashi (1994) has stated that at the international muscle biochemistry symposium held in Massachusetts in May of 1962 Annemarie Weber and he were essentially the only two participants who believed in the role of Ca^{2+} in contraction and relaxation. One of the main criticisms directed against the Ca^{2+} concept was that Ca^{2+}, while effective on a crude system such as glycerinated fibers or natural actomyosin, was not effective on reconstituted

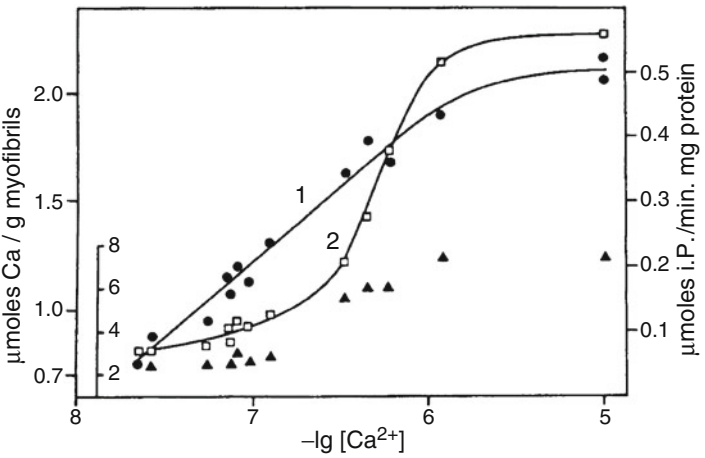

Fig. 4.15 Ca^{2+} binding, ATPase activity and superprecipitation of myofibrils as a function of ionized calcium concentration. Ordinates: *left*, Ca^{2+} binding (*curve 1*); *inset left*, superprecipitation (*triangles*); *right*, ATPase activity (*curve 2*). Abscissa, log of the free Ca^{2+} concentration. Note that Ca^{2+} binding to the myofibrils in the submicromolar range leads to acceleration of ATPase activity and superprecipitation (Weber and Herz 1963. With permission The American Society for Biochemistry and Molecular Biology)

actomyosin which was composed of separately prepared actin and myosin and was much purer than natural actomyosin. Basically it was asserted that a factor that was unable to act on a pure system could not be of vital importance (Ebashi 1980).

4.8 Discovery of the Ca^{2+} Receptor in Muscle Activation: Troponin

Ebashi and his colleagues took up this challenge in a series of remarkable papers, mostly "letters to the editor" from 1963 to 1968. The end result was the discovery and characterization of a new protein that Ebashi called troponin, the first intracellular Ca^{2+} binding protein. The first letter went to Nature and the next five letters were sent to the Journal of Biochemistry. The Journal of Biochemistry is an English language journal published in Tokyo on behalf of the Japanese Biochemical Society. Ebashi published most of his papers throughout the 1960s in this journal. One can suppose that there were at least two reasons for this approach. First, publishing significant papers in a Japanese journal with international distribution certainly promoted Japanese biochemistry and this was very important to Ebashi. Furthermore, letters to the editor were rapidly published. Ebashi could submit a letter in April and it would appear in print in June of July. Of course this was important in establishing priority in a rapidly moving field.

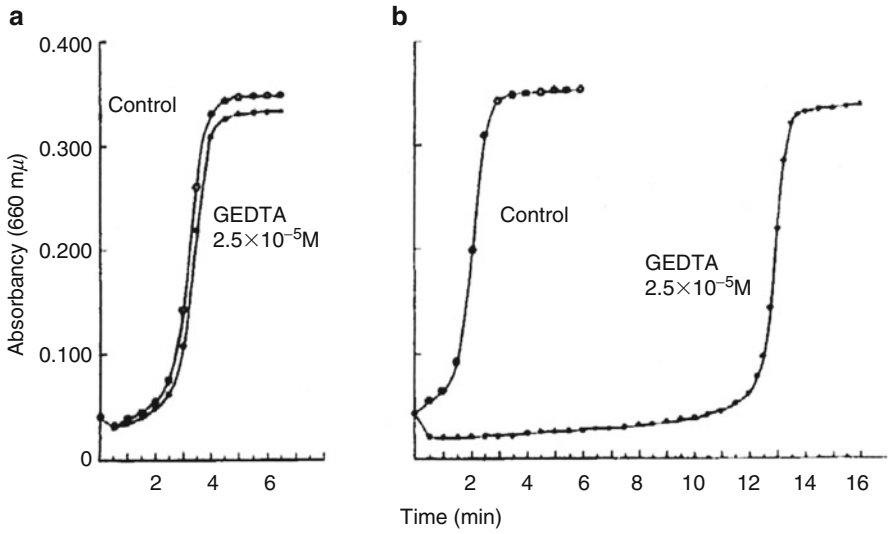

Fig. 4.16 Effect of tropomyosin-like protein on the superprecipitation of "natural actomyosin". Natural actomyosin (*control*) was made Ca^{2+} insensitive by treatment with trypsin. Superprecipitation was measured as an increase in turbidity versus time and taken as a measure of contraction. (**a**) Effect of GEDTA (EGTA) on the trypsin-treated natural actomyosin. (**b**) Effect of GEDTA on a mixture of trypsin-treated natural actomyosin plus purified tropomyosin-like protein. Note that the addition of tropomyosin-like protein changes the preparation from Ca^{2+} insensitive (**a**) to very Ca^{2+} sensitive (**b**) (Ebashi 1963. With permission Nature Publishing Group)

In a letter to Nature in 1963, Ebashi (1963) noted that a preparation of natural actomyosin isolated from rabbit skeletal muscle made Ca^{2+} insensitive by treatment with trypsin (Fig. 4.16a) could be made Ca^{2+} sensitive again, i.e., superprecipitate, by addition of a solution containing a "tropomyosin-like protein" to the mixture (Fig. 4.16b). This tropomyosin-like protein solution, also called native tropomyosin by Ebashi, exhibited all the properties of the tropomyosin protein discovered by Kenneth Bailey[8] at University of Cambridge in 1946. Tropomyosin is a very elongated fibrous protein associated with the myofibrils. While not derived from myosin itself, it has an amino acid composition similar to myosin. Bailey (1946) thought that tropomyosin might be a precursor or prototype of myosin, possibly even a subunit of myosin, and that is the reason for its name.

[8] Kenneth Bailey (1909–1963) spent most of his research career in the biochemistry department at the University of Cambridge. Beside his own discoveries relating to tropomyosin and invertebrate paramyosin (Bailey 1946), important discoveries were made by people training in his laboratory. Tien-chin Tsao (Tianqin Cao) discovered the subunit nature of the myosin molecule (see Chap. 3). B. B. Marsh discovered the 'relaxing factor' of muscle. Bailey also trained scientists who would become well known in the muscle field, including S. V. Perry (see Chap. 3) and J. C. Ruegg. Bailey was elected to a Fellowship at Trinity College in 1948 and to the Fellowship of the Royal Society in 1953. Despite his scientific success, he experienced and was treated for recurrent attacks of acute depression that worsened over the years. Tragically, he committed suicide in 1963 (Huxley 2007). Chibnall (1964) has written a biographical sketch of Bailey.

But native tropomyosin was somehow different from tropomyosin prepared according to the Bailey procedure because Bailey's tropomyosin did not restore Ca^{2+} sensitivity to the trypsin-treated natural actomyosin or synthetic actomyosin system. Some protein along with tropomyosin must be in the solution. In 1964, Ebashi and his wife Fumiko Ebashi described the properties and actions of this unnamed "new protein" in greater detail (Ebashi and Ebashi 1964). They simply referred to it as a "new protein component" that participated in the superprecipitation of synthetic actomyosin or trypsin-treated natural actomyosin. In a letter to the editor of the Journal of Biochemistry in 1965, Ebashi and Kodama (1965) for the first time reported the separation of the "tropomyosin-like protein" into two different components. In another brief letter to the editor of the same journal in 1966, Ebashi and Kodama (1966) gave this new protein the name "troponin" and showed that troponin interacted with F-actin but only in the presence of tropomyosin. Thus the tropomyosin-like protein or native tropomyosin consisted of tropomyosin and troponin and these proteins together bound to F-actin and restored Ca^{2+} sensitivity to synthetic actomyosin preparations.

Much more needed to be known about tropomyosin and troponin and their location and function in the myofibril. Endo et al. (1966) in yet another letter to the editor determined the location of native tropomyosin on myofibrils. Myofibrils isolated from rabbits were exposed to trypsin in order to digest tropomyosin and troponin. These myofibrils were then incubated with fluorescently labeled tropomyosin and troponin and observed by phase contrast and fluorescence microscopy. The results suggested strongly that the tropomyosin and troponin were located along the entire length of the thin filaments. These results were confirmed by fluorescent antibody staining of tropomyosin and troponin. In a fourth letter to the editor, Ohtsuki et al. (1967) showed in an immuno-electron microscopic study that antibodies to troponin were distributed at 400 Å intervals along the entire thin filament with 24 periodicities counted on either side of the Z line.

In the final letter to the editor in this series, Ebashi et al. (1967) proved that troponin was the calcium receptive protein in the model contractile systems in a very imaginative way. Strontium (Sr^{2+}) was known to substitute for Ca^{2+} in activating skeletal and cardiac natural actomyosin systems but with a lower potency than Ca^{2+}. The concentration of Sr^{2+} necessary for the activation of the skeletal muscle system was more than 20 times higher than that of Ca^{2+} but in cardiac muscle, it is only 3–4 times higher. All four proteins, myosin, actin, tropomyosin and troponin, were purified from both skeletal and cardiac muscle. All possible combinations were tested to determine the relative sensitivity to Sr^{2+} versus Ca^{2+}. Irrespective of the origin of the other three proteins, if troponin was prepared from skeletal muscle, the relative Sr^{2+} sensitivity of the reconstituted actomyosin system was low, but if prepared from cardiac muscle, it was high. These results clearly indicated that troponin was the site of the regulatory action of Ca^{2+} or Sr^{2+}. They concluded (Ebashi et al. 1967. With permission Oxford University Press):

> It is conceivable that binding and detaching of Ca^{++} to and from troponin might be of primary importance in regulation of muscle contraction, i.e., some conformational change of the troponin molecule induced by the removal of Ca^{++} might inhibit interaction of adjoining

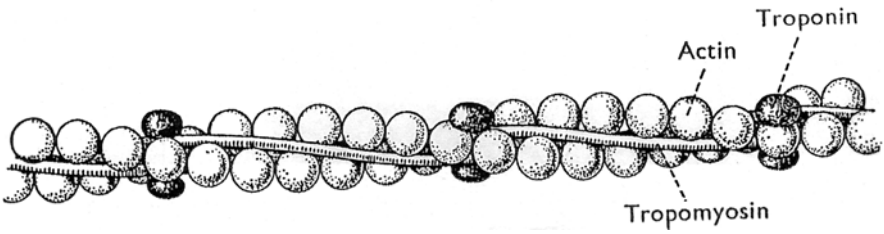

Fig. 4.17 A proposed model for the structure of the thin filament. In this model it is assumed that two molecules of tropomyosin and troponin exist in each period of the thin filament. The pitch of the double helix in the thin filament formed by the actin molecules is considered to be 360–370 Å, which is slightly shorter than the period due to troponin (Ebashi et al. 1969. With permission Cambridge University Press)

> actin molecule with myosin, and this inhibition might be cancelled by Ca^{++}. However, before accepting this explanation, we must answer the question as to how the troponin molecules, which are distributed along the thin filaments at a 400 Å periodicity (5), can exert their influence on those actin molecules which are located at some distance from adjacent two troponin molecules.

In 1968, Ebashi, Kodama and Ebashi published a full description of troponin, its preparation and physiological function, based mostly on the preliminary reports already described. They estimated that a troponin molecule bound about four Ca^{2+} with half of the binding of a high affinity in the submicromolar range and the other half with a 25-fold lower affinity. Tropomyosin did not bind Ca^{2+}. They emphasized that troponin alone could not restore Ca^{2+} sensitivity to Ca^{2+}-insensitive actomyosin preparations but rather also required tropomyosin. Furthermore, based on their relative molecular weights and content in the myofibrils, they speculated that it was plausible that two troponin molecules and two tropomyosin molecules exist per pitch of 400 Å of the thin filament. Ebashi et al. (1969) proposed a model for the structure of the thin filament (Fig. 4.17). In conclusion, Ebashi et al. (1968) stated that the results supported the hypothesis that troponin was the Ca^{2+}-receptive protein of the contractile system, i.e., the contraction-triggering action of Ca^{2+} became effective only when it bound to troponin. Thus the first intracellular Ca^{2+} receptor protein was discovered and its potential role in muscle contraction and relaxation proposed.

Hartshorne and Mueller (1968) at Carnegie-Mellon University went on to show that troponin could be fractionated into two distinct components. But Marion L. Greaser and John Gergely in 1971 at the Boston Biomedical Research Institute discovered that troponin was formed by three components in equimolar ratios (see Chap. 6). In 1972 Ebashi agreed with Greaser and Gergely that troponin is functionally composed of three components. Much more would be learned in the ensuing years about troponin and its components but the framework for the role of Ca^{2+} in muscle activation now was established.

4.9 Mechanism of Relaxation: The Mysterious "Relaxing Factor"

To many people, Setsuro Ebashi is best remembered for the discovery of troponin and characterization of its function. But Ebashi (1990) has said that the discovery of troponin was a natural consequence of the "calcium concept" and could have been done by any scientist, "perhaps even more elegantly." It was the development of the "calcium concept" and his publication of it in 1961 (Ebashi 1961a) that Ebashi considered to be his most important work. He has stated that this article was the culmination of nearly 10 years of inquiry into the mechanism of the relaxing factor and would remain "indelibly imprinted" in his memory" (Ebashi 1990).

The story of the relaxing factor began in 1951 with a discovery by a meat scientist working at the Low Temperature Station at Cambridge on a project designed to improve the quality of whale meat for human food (Marsh 1966). That scientist was B. B. Marsh from New Zealand who did the investigation in Kenneth Bailey's laboratory at the University of Cambridge. Whale meat wasn't available so Marsh (1951, 1952) examined, in the centrifuge, the effects of ATP on the time course of the decrease in volume of fragmented fibers in a homogenate of rabbit skeletal muscle. As a control for centrifugal packing of the fiber fragments, the time course of the volume change in the absence of ATP (rigor) was measured and subtracted from the time course of volume change in the presence of ATP (Fig. 4.18). When this correction was made, a striking and fundamentally important result was observed (Fig. 4.18, lower panel). The volume of the fiber fragments in the homogenate first increased, then became stable and finally slowly decreased as the ATP was exhausted. This effect was reversible and could be repeated by further addition of ATP. In the process of examining what was responsible for the volume increase or swelling of fiber fragments, Marsh also did the experiment by replacing the supernatant with isotonic KCl. Now a very different result appeared. ATP immediately caused a profound shrinkage or syneresis of the fragmented fibers, reducing the volume beyond what would be expected by centrifugal packing alone. This effect was irreversible. Marsh went on to examine the fiber fragments in the microscope under these two experimental conditions. When shrinkage occurred the fiber fragments shortened and when swelling occurred the fragments lengthened. Thus there was something in the supernatant that caused swelling or fiber fragment lengthening, i.e., relaxation. This relaxing effect in the presence of supernatant could also be overcome by addition of 1–2 mM $CaCl_2$. He concluded that the results indicated the existence of a substance, soluble in 0.16 KCl solution, which must be intimately concerned in volume increase effects. He referred to this substance simply as the "factor". This factor was normally present in the muscle. Over time this factor became known as the relaxing factor or sometimes also called the Marsh factor. Bendall (1952), also at the Low Temperature Station at Cambridge, extended Marsh's observations to muscle fiber model systems, including glycerinated muscle fibers. He observed that a fiber bundle that shortened in response to ATP could be made to relax by addition of a preparation containing the relaxing factor and

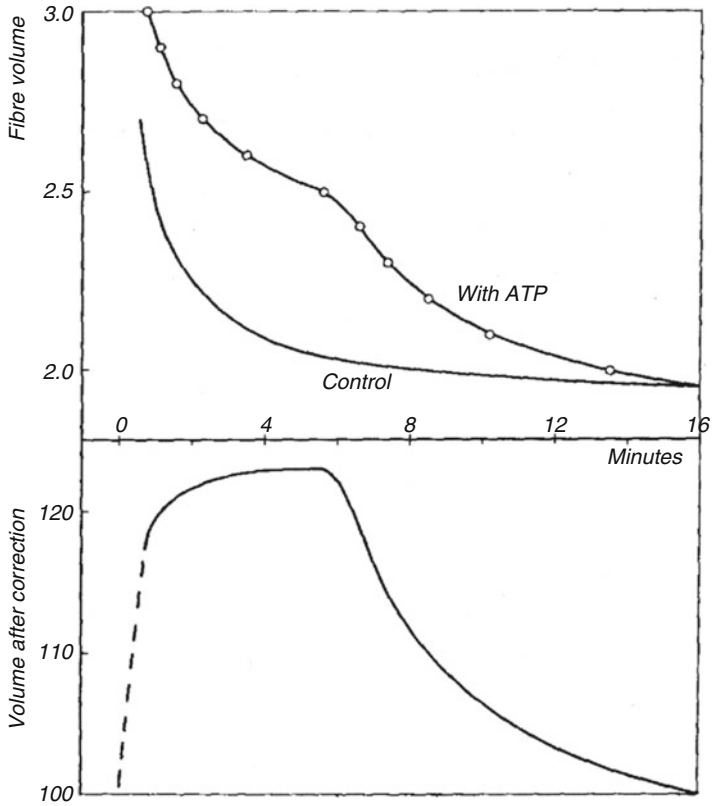

Fig. 4.18 The swelling effect of ATP on the volume of fiber fragments in a homogenate of rabbit skeletal muscle. *Upper panel*: time course of changes in fiber volume during centrifugation before (control) and after addition of ATP (at time zero) to a homogenate containing rigor fiber fragments. The decrease in volume of the rigor fragments (control) represents the effect of centrifugal packing. *Lower panel*: volume change of ATP containing fragments after subtracting the centrifugal packing curve. Note that the initial effect of ATP is to cause a swelling of the fiber fragments, i.e., a relaxing effect (Marsh 1952. With permission Elsevier)

ATP. Even though these observations were provocative, there would be missteps before the true nature of the muscle relaxing factor would be revealed almost 10 years later.

The next important step in the identification of the nature of the relaxing factor came in Hiroshi Kumagai's laboratory at the University of Tokyo. In 1955 Kumagai et al. (F. Takeda would later become Setsuro Ebashi's wife) showed that the relaxing factor was not myokinase or creatine phosphokinase as was previously proposed. Rather the active factor contained a considerable amount of lipid and exhibited a strong adenosine triphosphatase activity. It appeared to be similar to an ATPase bound to particulate matter that was discovered in 1948 by W. Wayne Kielley and

Otto Meyerhof at the University of Pennsylvania. In 1958 Ebashi concluded that the relaxation of glycerinated psoas muscle fibers required the presence of muscle granules and furthermore that the ATPase activity of the granules paralleled their effect on relaxation (Ebashi 1958).

As Ebashi was undertaking his studies of the relaxing factor during the 1950s, he was stimulated by Emil Bozler's observation in 1954 that the Ca^{2+} chelator, EDTA, imitated all the known effects of the relaxation factor. In extracted muscle fibers which have contracted in a solution containing ATP, the addition of EDTA caused relaxation, the subsequent addition of $CaCl_2$ contraction (Bozler 1954). As discussed above, Ebashi tested this Ca^{2+} concept by comparing the ability of chelators with varying affinities for Ca^{2+} to cause relaxation of glycerinated muscle fibers. The experimental results initially were disappointing as no correlation was observed (Ebashi et al. 1960). Somewhat discouraged, Ebashi went in late 1958 to spend a year in Fritz Lipmann's laboratory at the Rockefeller Institute in hopes of studying enzymology. Lipmann encouraged Ebashi to continue with his investigation of the relaxing factor. Just after his arrival, he realized his error in not considering the Mg^{2+} binding affinity of the Ca^{2+} chelators. Once corrected the correlation between relaxation and Ca^{2+} binding was excellent (Fig. 4.13). Also at this time Annemarie Weber (1959) discovered that small amounts of Ca^{2+} were necessary for contraction of myofibrils. The pieces were starting to come together.

Ebashi thought that the relaxing factor might be an ATP dependent Ca^{2+} chelator. He set out to test this hypothesis in Lipmann's laboratory. These experiments were greatly facilitated by the recent introduction of radio isotopes into biology in the form of Ca^{45}, C^{14}, P^{32}. His results were clear cut. The particulate fraction isolated from homogenized rabbit skeletal muscle that contained ATPase activity and produced muscle fiber relaxation was found to concentrate radioactive Ca^{2+} (Ca^{45}) up to 1,400-fold in an ATP dependent manner (Fig. 4.19) (Ebashi and Lipmann 1962). Ebashi (1985) has stated that this "cherished" experiment was the only experience in his life where the result completely lived up to his expectation. Furthermore George Palade[9] examined the particulate preparation in the electron microscope and concluded that the preparation contained resealed fragments or vesicles of the sarcoplasmic reticulum that he and Porter characterized in 1957 (Porter and Palade 1957). Muscatello et al. (1961) also showed that the microsomes utilized in relaxation experiments were fragments of the sarcoplasmic reticulum.

As a result of this work, Ebashi in 1961 put forward for the first time the "calcium concept" of muscle contraction and relaxation. Ebashi (1961a) described his evidence that Ca^{2+} activated the contractile proteins and that the fragmented vesicles of the sarcoplasmic reticulum induced relaxation by binding Ca^{2+} in the presence of

[9] In the Ebashi and Lipmann (1962) paper, George Palade performed the electron microscopy of the particulate preparation that accumulated Ca^{2+}, interpreted the electron micrographs, wrote the related methods and legends but yet was not a co-author on the paper. This is very unusual by modern day standards, especially since the identification of the particulate preparation as fragmented sarcoplasmic reticulum was an important finding.

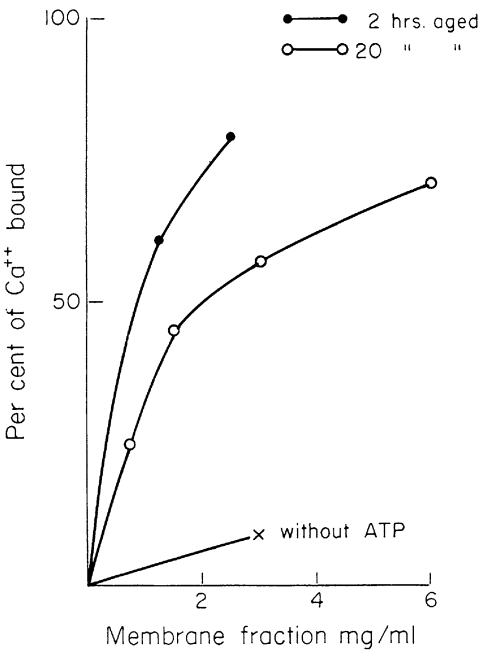

Fig. 4.19 Influence of membrane fraction (fragmented sarcoplasmic reticulum) on the ATP dependent Ca^{2+} concentrating reaction. An aged membrane fraction was less effective in accumulating calcium (Ebashi and Lipmann 1962. With permission Rockefeller University Press)

ATP. Ebashi was most proud of this paper because it was the result of 10 years of investigation and led to the development of the calcium concept. Ebashi concluded (Ebashi 1961a. With permission Oxford University Press):

> …we suggest the following picture of muscle contraction. In resting muscle calcium is highly concentrated in the endoplasmic reticulum; the concentration of calcium inside the myofibril is too low to cause the shrinking of contractile protein. When the muscle is excited, the concentrated calcium in some portion of the endoplasmic reticulum is released by the electrical influence due to the depolarization of the muscle membrane, and the calcium thus released causes in turn the shrinking of the actomyosin system. When the excitation is over, calcium release ceases and the liberated calcium is recaptured by the endoplasmic reticulum (the relaxing factor); consequently the shrunken actomyosin-system is restored to its relaxed state.

A second paper in 1961 (Ebashi 1961b) was a review of the results leading to the calcium concept. Thus the calcium concept of muscle contraction and relaxation was born. Much work would need to be done to prove the correctness of the hypothesis.

On the surface of it everything seemed fine but there was a problem for the respectful Ebashi who had the honor of working in the laboratory of a Nobel Laureate. Lipmann was slow to believe that anything as simple as Ca^{2+} could control muscle contraction and relaxation. Ebashi conducted the experiments in 1959 but the paper wasn't received by the Journal of Cell Biology until April of 1962. Thus there is an unusual statement in the introduction to the paper (Ebashi and

Lipmann 1962) indicating that the work was done about 3 years previously but, "due to various circumstances", publication was delayed. The reason for this statement was that Wilhelm Hasselbach[10] and Madoka Makinose (see footnote 10) at the Max Planck Institute for Medical Research in Heidelberg reported similar results a year earlier (Hasselbach and Makinose 1961).

Hasselbach started working on the identity of the relaxation factor with Hans H. Weber in 1953 (Hasselbach 1989). They found that the relaxing factor effectiveness was enhanced in the presence of the Ca^{2+} binding agent oxalate. Annemarie Weber (Hans Weber's daughter) suggested that he determine if removal of the microsomes from their preparation by centrifugation would affect relaxing activity. He confirmed Ebashi's findings and found that relaxing activity was present in the microsomes. In 1961; 1962 and 1963, Hasselbach and Makinose published a series of classic papers describing the relationship between Ca^{2+} uptake by the microsomes and ATP hydrolysis. Their results were very similar to those of Ebashi and Lipmann (1962). Hasselbach and Makinose discovered that oxalate which diffused into the microsomal vesicles could greatly increase Ca^{2+} uptake by the vesicles by forming a complex with Ca^{2+} in the vesicles and thus reducing the free Ca^{2+} concentration inside the vesicles. The ability of microsomes to remove Ca^{2+} from the medium depended on the presence of ATP and Mg^{2+} but Mg^{2+} was not transported into the vesicles. They calculated that the free Ca^{2+} concentration was higher in the mircosomes than in the medium. Thus the Ca^{2+} is transported against a concentration gradient. They boldly concluded that the microsomes function as a Ca^{2+} concentrating system, i.e., the calcium pump (Die Calciumpumpe), the energy for the pump being derived from ATP (Hasselbach and Makinose 1961).

Ebashi and Lipmann (1962) found that the hydrolysis of ATP by the vesicles was accompanied by an exchange of ATP and ADP. This ATP-ADP phosphate exchange reaction was interpreted as indicating a reversible phosphorylation of the membrane: ATP + membrane ↔ ADP + membrane-P. In 1962 Hasselbach and Makinose correlated for the first time the time course of Ca^{2+} uptake and ATP hydrolysis with the rapidly proceeding transfer of the terminal phosphate of ATP to ADP. The activation of this exchange activity of the transport system by Ca^{2+} was the first indication of the occurrence of a phosphorylated intermediate in a transport reaction cycle. Hasselbach and Makinose (1962) speculated that the unknown substance became a

[10] Wilhelm Hasselbach (b.1921) received his M.D. from the University of Marburg in 1949 after being released as a prisoner of war by the US Army, first Infantry Division early in 1945. He became a member of H. H. Weber's laboratory and published an influential paper on selective extraction of myosin form myofibrils in 1951. He then moved to Heidelberg from Tubingen with H. H. Weber in 1954. In 1964 he succeeded Weber as director of the Max Planck Institute for Medical Research in Heidelberg. He received the Feldberg Prize in 1963 for his work establishing the calcium pump of the sarcoplasmic reticulum. He retired from the Max Planck Institute in 1990. Madoka Makinose first joined Weber's research group in 1957 as a postdoctoral fellow from Sapporo Medical College in Japan. He returned to Germany permanently in 1960 and played a pivotal role with Hasselbach in the development of the evidence for the calcium pump. He continued to collaborate with Hasselbach and also worked independently on various aspects of calcium pump function with his last paper appearing in 1992.

"carrier by phosphorylation" and that the Ca^{2+} complex of the phosphorylated carrier diffused to the inner surface of the membrane of the vesicles and there the phosphate group was split off from the carrier leading to a large decrease in Ca^{2+} affinity of the carrier. These results were confirmed and extended by Yuji Tonomura (1923–1982) and Taibo Yamamoto (Yamamoto and Tonomura 1967) at Osaka University. From their study, especially of the phosphorylation kinetics, they developed the first reaction schemes for the calcium pump. The sarcoplasmic reticulum calcium pump is now classified as a P-type ion pump or P-ATPase since it functions via the formation of a phosphorylated intermediate. Some other P-ATPases include the Na,K-ATPase and the Ca-ATPase of the plasma membrane.

In 1963 Hasselbach and Makinose revised their earlier estimate of the stoichiometry of the calcium pump and now concluded that stoichiometry was two Ca^{2+} transported per one ATP hydrolyzed. Weber et al. (1966) verified this conclusion and extended it over a wide range of Ca^{2+} concentrations.

When Hasselbach visited the Rockefeller Institute in 1962, he "encountered fierce criticism" from Lipmann and his co-workers (Hasselbach 1989). They objected to the use of oxalate as a Ca^{2+} trapping agent. They did not believe that Ca^{2+} was accumulated by an ATP supported pump but preferred an ATP dependent Ca^{2+} binding process. Hasselbach was unaware of Ebashi's results "which were still in one of Lipmann's drawers". The findings stayed in "Lipmann's drawer" for nearly 2 years.

An important unaddressed question was could the calcium pump operate rapidly enough to explain the fast relaxation observed in muscles? Ebashi thought that the pump was not fast enough to explain relaxation and he thus was thinking in terms of an ATP dependent Ca^{2+} binding to the vesicles in analogy with the effects of calcium chelators. He did a time resolved spectrophotometric investigation of rate of Ca^{2+} binding to the vesicles (Ohnishi and Ebashi 1964) and found that in the presence of Mg^{2+} and ATP, Ca^{2+} was very rapidly bound to the relaxing vesicles. They concluded that "for the time-being" it was most plausible to suppose that the main part of Ca^{2+} was bound to the surface of sarcoplasmic reticulum (surface which is in contact with sarcoplasm) and only a minor part is accumulated inside the vesicles. Ebashi thought that the conclusions of Hasselbach and Makinose were dependent on the non-physiological action of oxalate. In the end, Hasselbach and Makinose would be shown to be correct as the results of Ohnishi and Ebashi were not confirmed by other investigators. Furthermore by comparing the rate of Ca^{2+} pumping by vesicles of the fragmented sarcoplasmic reticulum with the rate of frog muscle relaxation at low temperature, Weber et al. (1966) concluded that the speed of the Ca^{2+} uptake by reticular transport system in skeletal muscle was adequate to explain the rate of relaxation within a factor of 2.5. This agreement is reasonable given the fact that the relaxation of an intact muscle was compared to Ca^{2+} uptake by isolated vesicles of the sarcoplasmic reticulum. But in the long run more direct evidence would be needed.

Hasselbach and Makinose also made a mis-step in their interpretation. Because they knew that ATP induced superprecipitation of purified actomyosin was not affected by Ca^{2+}, they (Hasselbach 1989) developed the working hypothesis that in

presence of ATP the microsomes produced a "soluble calcium-sensitive relaxing factor". They tried to incorporate their view of the calcium pump into this hypothesis. In the early 1960s, other laboratories also were pursuing the hypothesis that the sarcoplasmic reticulum produced a "soluble relaxing factor". It was soon after the discovery of cyclic AMP by Earl W. Sutherland in 1958 (Kresge et al. 2005) and many investigators felt that the soluble relaxing factor would turn out to be a complex organic molecule. For example, Laszlo Lorand (1964) speculated that the membrane system of the sarcoplasmic reticulum might act as a "metabolic assembly synthesizing a diffusible relaxation substance" that would somehow prevent the contractile process on the myofibrils from occurring.

Hasselbach (1989) has stated that he abandoned this idea of a soluble relaxing factor after seeing the results of Weber et al. (1963). They determined that the relaxing factor could remove Ca^{2+} from actomyosin or myofibrils to an extent sufficient to explain the relaxing effect. Furthermore the vesicles of the relaxing factor were capable through the mechanism of Ca^{2+} accumulation of reducing the outside concentration of Ca^{2+} to such low levels that the equilibrium between Ca^{2+} and actomyosin was shifted toward dissociation. Finally the time course of Ca^{2+} removal paralleled the time course of inhibition of ATPase and reversal of syneresis. With regard to the effects of the relaxing factor, they concluded that it was not necessary to postulate any additional mechanism—such as a soluble relaxing substance—to explain its effect on actomyosin systems (Weber et al. 1963). The concept of the soluble relaxing factor finally became obsolete when Ebashi discovered the Ca^{2+} binding protein troponin.

The next important step was to couple the biochemical results regarding the role of Ca^{2+} in contraction and relaxation with muscle structure. The biochemical results showed that Ca^{2+} could be sequestered in the fragmented sarcoplasmic reticulum. Where was Ca^{2+} located in the resting muscle fiber? To answer this question, Costantin et al. (1965) utilized a frog muscle fiber preparation developed by Reiji Natori[11] (1954; 1986) in which the surface membrane was peeled off in paraffin oil leaving the intracellular components intact. This muscle fiber preparation is called a mechanically "skinned" muscle fiber. Following a recommendation by Andrew Huxley (Podolsky 1989), they perfused this resting "skinned" fiber with a Ca^{2+} solution containing oxalate in order to increase the participation of Ca^{2+} and to make it easier to locate calcium deposits in the electron microscope. They found that electron-opaque material, probably calcium oxalate, accumulated in the terminal sacs of the sarcoplasmic reticulum. They concluded that these regions of Ca^{2+} accu-

[11] Reiji Natori (d. 2006) graduated from the Jikeikai School of Medicine in 1936 and spent his entire career in the department of physiology at the Jikeikai School of Medicine where he became a professor emeritus in 1977. He developed the skinned muscle fiber preparation in 1949. Natori (1986) has given a chronological account of his studies utilizing skinned muscle fibers. In the mechanically skinned muscle fiber, the surface membrane has been removed allowing access to the fiber interior which still contains the structural and biochemical components of the intact fiber. In contrast, in the glycerinated muscle fiber preparation developed by Albert Szent-Gyorgyi not only the surface membrane but also the intracellular membranes have been destroyed and soluble proteins removed.

mulation represented the intracellular Ca^{2+} sink that controlled the relaxation phase of the contraction-relaxation cycle. Furthermore they noted that their proximity to the transverse tubules which were implicated in intracellular stimulus conduction suggested that they might also be regions from which Ca^{2+} was released to trigger contraction. Thus the biochemical results and muscle structure were brought together in a most satisfying way. By the mid- to late nineteen sixties, the "calcium concept" had become well established. Ebashi and Endo (1968) and Weber (1966) have written excellent reviews covering this important, exciting and sometimes confusing period of research.

Thus during the development of the calcium concept of muscle contraction and relaxation three "giants" dominated the stage. Setsuro Ebashi was the first to articulate the calcium concept which guided research during this period. Annemarie Weber was the first to show that Ca^{2+} was required for contraction of model muscle systems. Wilhelm Hasselbach (and Makinose) was the first to prove that a calcium pump caused the effects of the relaxing factor. Finally, Ebashi was the first to discover the myofibrillar calcium receptor troponin.

4.10 The Ca^{2+} Transient: Contraction and Relaxation in Muscles

Despite the spectacular advances made by the biochemists, it was still crucial to prove in muscles that the proposed changes in free Ca^{2+} concentration during contraction and relaxation actually conformed to the calcium concept. Some of the important research in this regard in the 1960s was performed on unconventional muscle preparations, namely giant muscle fibers isolated from invertebrates. Peter Caldwell (1927–1979), of Bristol University, regularly worked at the Laboratory of the Marine Biological Association in Plymouth, England and had collaborated with Alan Hodgkin and Richard Keynes. Thus he was well aware of the experiments where substances where injected into the interior of squid axons through cannulation of the axon. He reasoned that that same procedure would be possible with the giant muscle fibers from the spider crab, *Maia squinado*. These fibers could reach diameters of 1–2 mm. He and Walster (1963) developed just such a muscle preparation. They were able to microinject various substances through a cannula into the muscle fiber and then insert an electrode longitudinally into the fiber for transmembrane potential measurements. Their results were reminiscent of those of Heilbrunn and Wiercinski in 1947 in that of the ions injected only Ca^{2+} gave a substantial contraction of the fiber. Hildegard Portzehl from the University of Bern and Johann Caspar Ruegg from the Max Planck in Heidelberg joined Caldwell at the Plymouth laboratory in 1963. They injected solutions containing low concentrations of Ca^{2+} stabilized by EGTA into the giant fibers (Portzehl et al. 1964). The goal was to determine the threshold concentration of free Ca^{2+} that was needed to cause contraction in an intact muscle fiber. The Ca^{2+}-EGTA buffers which just caused contraction

contained 0.3–0.7 μM free Ca^{2+}. Thus the free Ca^{2+} concentration in resting muscle fibers must be lower than these values. These results were in good agreement with those of Annemarie Weber (Weber and Herz 1963) on isolated myofibrils and purified actomyosin. These experiments were an important step in linking the biochemistry with the physiology of muscle contraction. In 1965 Christopher C. Ashley,[12] who did his doctoral research with Peter Caldwell, showed that intracellular injections of EGTA into single cannulated muscle fibers were able to suppress, almost completely, the contractions induced by various contractile agents such as caffeine, high K^+ or electrical stimulation (Ashley et al. 1965). Richard J. Podolsky (1923–2001) had just finished postdoctoral training at University College London and was now at the NIH Naval Medical Research Institute. He applied Ca^{2+} to the Natori skinned muscle fiber preparation in oil through a micropipette with a tip diameter of 1 μm (Podolsky 1964). He found that this local application of Ca^{2+} resulted in a local contraction but only if the sarcomere length was less than 3.7 μm. Since this critical sarcomere length for contraction in response to added Ca^{2+} was very close to the length found by Huxley and Peachey (1961) for activation by electrical stimulation of an intact muscle fiber, it was reasonable to conclude that that electrical stimulation was followed by Ca^{2+} release. Thus the evidence for the calcium concept was moving from the biochemistry of actomyosin and isolated myofibrils into the realm of intact muscle fibers.

But what was really needed was a direct measure of the expected time course of changes in intracellular free Ca^{2+} concentration with muscle contraction. The first investigators to attempt to measure the so-called calcium transient in intact muscle were Frans F. Jobsis (1929–2006) and medical student Michael J. O'Connor at Duke University (1966). They introduced the Ca^{2+} sensitive dye murexide into toads by repeated intraperitoneal injection and observed changes in murexide absorbance in the isolated sartorius muscle in response to electrical stimulation. Because murexide is a dye that undergoes absorbance changes upon binding Ca^{2+}, they attributed the optical signals to calcium transients. They did not follow up these preliminary observations with a complete study at least in part because of the difficulty in getting murexide reproducibly into the muscle.

The first comprehensive investigation of intracellular calcium transients in intact muscle was performed by Ashley and Ellis Ridgway (see footnote 12) at the University of Oregon. These interesting and imaginative studies represent literally

[12] Christopher C. Ashley (1941–) received his Ph.D. from Bristol University under the guidance of Peter Caldwell. After 3 years as a Fulbright Scholar in the United States, he returned to the department of zoology at the University of Bristol. He eventually moved to the university laboratory of physiology in Oxford (now the department of physiology, anatomy and genetics, University Oxford). Throughout his career he has investigated various aspects of the regulation of muscle contraction by Ca^{2+} with publications that span more than 40 years. Ellis B. Ridgway received his Ph.D. in biology (1968) from the University of Oregon under Graham Hoyle. Following postdoctoral fellowships in London with Andrew Huxley and in Cambridge with Alan Hodgkin, Dr. Ridgway joined the department of physiology and biophysics at the Medical College of Virginia (now the Virginia Commonwealth University) in 1972. His research has revolved around assessing the role of Ca^{2+} ion in a number of biological phenomena.

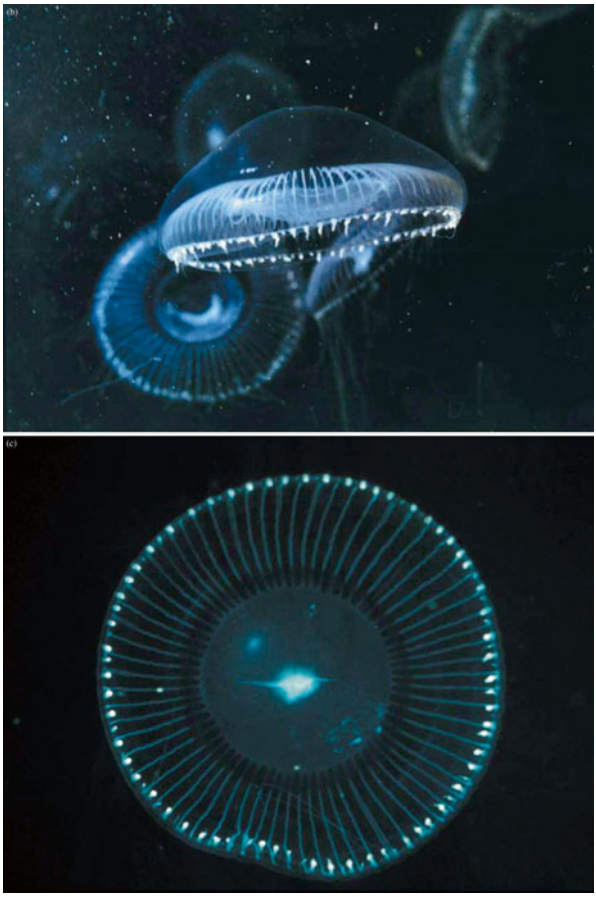

Fig. 4.20 The jellyfish, *Aequorea forskalea*, and its bioluminescence. *Top*: The light emitting cells of the jellyfish are located in the edge of the umbrella at the base of the tentacles. *Bottom*: *Aequorea* forskalea emits a greenish light upon agitation. During the light emission, blue light of the aequorin molecules is converted to green light by a cofactor, the green fluorescent protein (GFP) (Shimomura 2005. With permission John Wiley & Sons Inc)

one of the more "colorful" chapters in the history of muscle research. The story begins not with muscle at all but with the discovery of a photoprotein by Osamu Shimomura, Frank H. Johnson and Yo Saiga in 1962 at Princeton University. Shimomura, on leave from the University of Nagasaki, came to the biology department at Princeton University which was at the time the "mecca" for the study of bioluminescence. The world's leading authorities were there including E. Newton Harvey and Frank Johnson. They investigated the mechanism of the luminescence of the jellyfish, *Aequorea aequorea* (also referred to as *Aequorea victoria* or *Aequorea forskalea* after its discoverer Peter Forskal in 1775 [Harvey 1957]) which could be collected in abundance at Friday Harbor in the state of Washington. The jellyfish are nearly transparent and will only flash when stimulated or agitated (they don't sting) and then they emit a greenish luminescence. The jellyfish are shaped like a hemispherical umbrella and the light emitting organs are located along the edge of the umbrella at the base of the tentacles (Fig. 4.20). From these organs they isolated a protein that emitted a bluish light in response to very low levels of

Ca^{2+}. About a thousand jellyfish had to be collected with hand nets to yield, upon purification, 0.5 mg of protein. They called this photoprotein, aequorin.[13] Before aequorin was discovered bioluminescent systems were thought to be all of the luciferin (the substrate)/luciferase (the enzyme) type. In contrast aequorin emitted light in response to Ca^{2+} only and did not require an enzyme. Thus aequorin was a new type of bioluminescent system, a photoprotein. In 1963 Shimomura, Johnson and Saiga suggested that aequorin might be utilized as a Ca^{2+} indicator for biological research.

Ashley and Ridgway took up this suggestion with the idea of measuring the calcium transient in a muscle fiber by measuring the time course of aequorin light emission. At about the time of the discovery of aequorin, Graham Hoyle and T. Smyth (1963) at the University of Oregon discovered giant muscle fibers (up to 2 mm in diameter) in the barnacle *B. nubilus*. Ashley was already familiar with cannulation of giant fibers and he and Ridgway applied this technique to the barnacle fibers while working with Hoyle's laboratory group. They injected aequorin into the fiber and then inserted an electrode in the fiber to measure transmembrane potential and then stimulated the fiber to contract. The barnacle muscle fibers do not normally produce an all-or-none action potential when stimulated. They produce graded responses upon electrical stimulation. What Ashley and Ridgway saw when the fiber was stimulated must have been tremendously exciting. The fiber produced a blue flash of light. This light was actually visible to the dark adapted eye! They measured the time course of light emission with a photomultiplier (Ridgway and Ashley 1967; Ashley and Ridgway 1968, 1970). In what has become a classic figure (Fig. 4.21), they measured simultaneously the graded electrical response, the calcium transient as the light emitted by aequorin and the isometric force developed by the muscle fiber. The onset of the calcium transient occurred just after the onset of membrane depolarization but before the onset of detectable force. The calcium transient peaked early in the mechanical response and was virtually complete at peak force and in somewhat of a surprise mechanical relaxation occurred at essentially resting calcium levels. They (Ashley and Ridgway 1970) concluded that the simplest explanation of this latter observation was that, while Ca^{2+} was required for the generation of tension, it was not required for the maintenance of tension since at peak tension Ca^{2+} was already re-accumulated by the SR during the exponential fall of the calcium transient. This simple interpretation, of course, leads to the conclusion that relaxation involves some process other than Ca^{2+} removal from the contractile machinery. This interpretation would turn out to be incorrect but the apparent result of relaxation occurring at resting levels of free Ca^{2+} concentration would

[13] Shimomura et al. (1962) also isolated another protein which they called "green protein", later known as "green fluorescent protein" or GFP from the same organism. GFP is not required for luminescence but serves as a wavelength converter, accepting energy that would otherwise be emitted as blue light and emitting green light in its place. It was the discovery of GFP by Shimomura, the subsequent utilization of its light emission to monitor gene expression by Martin Chalfie at Columbia University and the modification of the color of light emission of GFP that allowed the monitoring of several biological processes at the same time by Roger Y. Tsien at the University of California at San Diego that led the three to share the Noble Prize in chemistry in 2008.

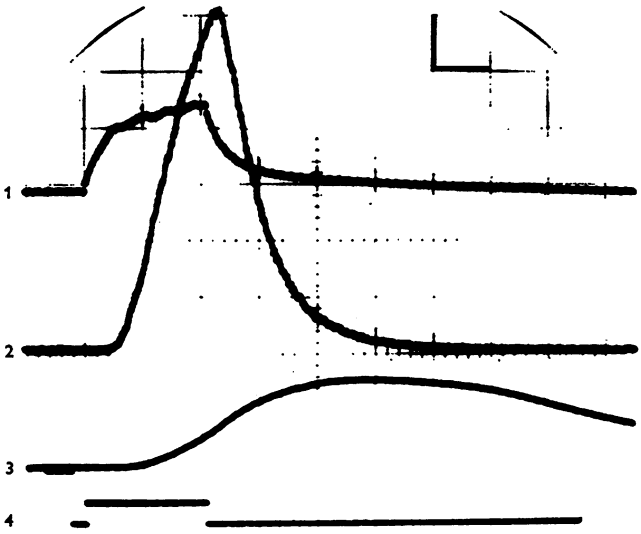

Fig. 4.21 Simultaneous recordings of membrane potential, calcium transient and isometric force in a single barnacle muscle fiber. Trace 1 is the graded membrane potential monitored by an intracellular recording electrode in response to a single depolarizing pulse of 200 ms duration. Trace 2 is the Ca^{2+}-mediated light emission from aequorin recorded by a photomultiplier. Trace 3 is isometric force. Trace 4 is a stimulus mark which indicates the onset and cessation of the stimulus pulse. Note the temporal sequence of events is from initiation of electrical activity to the increase in free Ca^{2+} concentration leading to force development (Ashley and Ridgway 1970. With permission John Wiley & Sons Inc)

require further investigation. They went on to perform a thorough study of the effects of stimulus strength, stimulus duration, repeated stimulation on the calcium transient and isometric tension. This was a powerful study in that it made it feasible to measure in muscle the temporal relationships of membrane potential, calcium transients and force production.

Since this study was done on an unconventional muscle preparation, there was interest in measuring calcium transients in the more usual preparations utilized for electrical and mechanical studies, such as frog and toad muscle fibers. In about 1966 John Blinks (1931–2012) at the Mayo Foundation took up this challenge (Blinks et al. 1976). It wouldn't be until 1973 that Blinks was able to report the results of his collaborators, postdoctoral fellows Reinhardt Rudel from Germany and Stuart Taylor from Alexander Sandow's laboratory at New York University (Blinks 1973). They recorded the time course of aequorin light emission in response to electrical stimuli of various frequencies, from single twitches to a fused tetanus in toad muscle fibers. These experiments were particularly difficult. Aequorin, a large molecule of molecular weight about 21,000 Da had to be pressure injected through a microelectrode into the fiber without damaging the fiber. Also it turned out that aequorin tracked the time course of the Ca^{2+} changes with a lag which was more of a problem the faster the calcium transient. Finally the aequorin response was non linear with

changes in free Ca^{2+}. [For a review of the technical issues relating to aequorin as a biological Ca^{2+} indicator, see Blinks et al. 1976.] There would be other interesting and important experiments conducted measuring calcium transients with aequorin in skeletal and cardiac muscle fibers. But eventually the use of aequorin as an indicator of changes in free Ca^{2+} concentration during muscle contraction would be replaced by indicators that were easier to obtain, easier to get into fibers, faster in response to changes of free Ca^{2+} and linear in response to changes in free Ca^{2+}. Measurement of changes in free Ca^{2+} concentration in muscle fibers has become a routine procedure today (see Chap. 8). Nonetheless the early experiments by Ashley and Ridgway and Blinks, Taylor and Rudel were seminal in opening up the field of calcium transient measurement in muscle fibers.

If the generalizations inferred from studies on subcellular systems are applicable to the intact muscle, exchangeable Ca^{2+} should be bound by troponin during contraction and thus one would expect to find exchangeable Ca^{2+} absent from the region of the thin filaments in resting muscle and present in significant amounts in contacting muscle. In the mid to late 1960s, Saul Winegrad in the department of physiology at the University of Pennsylvania built upon his postdoctoral work in the laboratory of Andrew Huxley. He was the first to attempt to measure the location of Ca^{2+} in a muscle at rest and during and after tetanic contraction. He developed autoradiographic techniques to measure the intracellular location of ^{45}Ca in frog skeletal muscles. He demonstrated that Ca^{2+} was located primarily in the region of the terminal cisternae in the resting muscle (Winegrad 1970). Sections of muscles rapidly frozen during contraction and then fixed were characterized by a decline in ^{45}Ca radioactivity in the region of the terminal cisternae and a rise in activity in the region of the sarcomere occupied by the thin filaments. Winegrad (1970) concluded that during a tetanus, most of the Ca^{2+} in the terminal cisternae shifted to the region of the thin filaments. Even though this technique did not have great spatial resolution and would be superseded later by elegant experiments using electron probe microanalysis (Somlyo et al. 1981), the results were consistent with the calcium concept.

4.11 The Later Years: Setsuro Ebashi

The 1960s were incredibly productive years for Ebashi and his collaborators. In 1965 Ebashi, his wife Fumiko Ebashi and Koscak Maruyama (Ebashi and Ebashi 1965; Maruyama and Ebashi 1965) announced the discovery of a new structural protein in muscle which they named α-actinin. Their laboratory subsequently localized this protein to the z-band (Masaki et al. 1967). Then Ebashi and collaborators discovered M-protein (Masaki et al. 1968) which was located at the M-line of the sarcomere. In 1967 Eijiro Ozawa, Keizo Hosoi and Ebashi discovered that Ca^{2+} in a concentration that activates muscle contraction also activated glycogenolysis by reversibly converting phosphorylase b to phosphorylase a.

Weber (1994) has summed up what she called Ebashi "great achievements". She emphasized that Ebashi had introduced all the major features of an intracellular

signaling system into biology: first, a target protein, troponin, for the intracellular messenger, Ca^{2+}; second, the concept that a messenger should coordinate cellular activities around a major task: in this case, switching on the energy supply for mechanical work, i.e., activation of phosphorylase kinase by the same Ca^{2+} concentration necessary for Ca^{2+} binding to troponin and third, a mechanism to terminate the message, ATP driven Ca^{2+} uptake by the sarcoplasmic reticulum. These were indeed great achievements.

Ebashi went on to investigate the regulatory proteins in cardiac and smooth muscle. He and his collaborators proposed that smooth muscle activation was initiated by Ca^{2+} binding to a protein that they called leiotonin, an analogue of troponin, which along with tropomyosin was associated with the actin filaments of smooth muscle (Ebashi et al. 1977). They proposed that the leiotonin-tropomyosin system was the regulatory factor common to all kinds of vertebrate smooth muscle. This idea never became accepted and it is now thought that smooth muscle is activated via a Ca^{2+} dependent myosin light chain kinase system as originally proposed by David J. Hartshorne and his colleagues (Aksoy et al. 1976). Even as late as 1995 Ebashi was still clinging to the idea of leiotonin's role in smooth muscle activation but it was not to be and leiotonin seems to have drifted quietly into the past. This apparent mis-step in no way lessens the great scientific achievements of Ebashi or his monumental role in promoting Japanese muscle biochemistry and the careers of Japanese scientists.

By the end of the 1960s much had been discovered about the role of Ca^{2+} in muscle contraction and relaxation. These discoveries included the development of the calcium concept, elucidation of the pathway of spread of the surface electrical activity into the fiber interior, location of Ca^{2+} at rest, measurement of the time course of changes in free Ca^{2+} concentration during contraction and relaxation, determination of the sensitivity of the contractile apparatus to Ca^{2+}, discovery of the role of troponin in activation of contraction, and the role of the sarcoplasmic reticulum calcium pump in sequestering Ca^{2+} and causing relaxation. But in an important sense this understanding wasn't truly molecular in nature. It was really a broad outline that would require elucidation of detailed molecular mechanisms to have a complete understanding of the role of Ca^{2+} in muscle contraction and relaxation. Many of these molecular mechanisms would be elucidated throughout the rest of the twentieth century.

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