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Review

Animal electricity, Ca^{2+} and muscle contraction. A brief history of muscle research

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"There is a dead literature, and there is a live one.
The dead is not all ancient, the live is not all modern."
Oliver Wendell Holmes

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This brief review attempts to summarize some of the major phases of muscle research from Leeuwenhoek's description of sarcomeres in 1674, through Galvani's observation of "animal electricity" in 1791, to the discovery of Ca^{2+} as the key messenger in the coupling of nerve excitation to muscle contraction. The emerging molecular mechanism of the contraction process is one of the great achievements of biology, reflecting the intimate links between physics, chemistry and the life Sciences in the solution of biological problems.

LEEUWENHOEK'S GLOBULES AND CROONE'S HYPOTHESIS OF MUSCLE CONTRACTION

Speculations about muscle motility have a long history and many theories of muscle contraction appeared over two millennia [1]. But the dawn of modern muscle research probably begins in 1674–1682 with the microscopic observation of cross-striations and myofibrils in muscle fibers by Leeuwenhoek [1]. He wrote [2]: "I could distinctly see that the fleshy fibers, of which the greater part of a muscle consists, were composed of globules." He presented a drawing, that clearly shows the cross striations which delineate the "globules" i.e., sarcomeres, and calculated that a muscle fiber may contain thousands of filaments. He wrote [3]: ". . .who can tell, whether each of these filaments may not be enclosed in its proper

★75th Anniversary of Membrane Lipid Bilayer Concept.

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membrane and contain within it an incredible number of still smaller filaments."

Based on these observations Croone suggested in 1675–1680 [4], that the sarcomeres ("globules") delineated by cross-striations may serve as units of contraction. He also assumed that contraction occurs without a change in muscle volume and proposed that nerves play a role in conducting the stimulus from the brain to the muscle fiber. With these observations Leeuwenhoek and Croone clearly set the stage for the next 300 years of muscle research.

THE DISCOVERY OF "ANIMAL ELECTRICITY". GALVANI AND VOLTA

The next major step a century later is the discovery of "animal electricity". Luigi Galvani observed in 1792 that frog muscle contracts vigorously when its nerve is touched with a scalpel during a spark discharge from a distant electric machine [5–7]. Contraction could also be produced by connecting the leg nerve to the muscle through a metal conductor. Galvani concluded that intrinsic "animal electricity" is present in the nerve and muscle, and contraction is induced by permitting the flow of this electricity through a conductor.

Alessandro Volta initially expressed admiration for these observations, but later attributed the contractions seen by Galvani to outside sources instead of animal electricity [7, 8], and initiated a fierce debate that lasted through Galvani's lifetime.

Galvani countered Volta's argument in 1794, showing that contractions can be produced simply by placing the tip of a sectioned nerve in contact with the muscle surface. In 1797 he further showed that when the surface of the sectioned right sciatic nerve touched the intact surface of the left sciatic nerve, both legs contracted [7]. Neither of these experiments was accepted by Volta as conclusive proof of animal electricity.

In explaining his observations Galvani proposed, long before the advent of cell theory, that muscle and nerve fibers must possess a mechanism for generating electricity by accumulating positive and negative charges on two opposite surfaces, in analogy with the internal and external surfaces of a Leyden jar. To account for the flow of electricity during excitation, he also assumed the existence of fluid-filled pores (the first hint at channels) between the internal and external surfaces, that serve as conducting pathways.

By varying the strength of electric discharge, Galvani showed that contractions required a minimal threshold stimulus, and increasing the stimulus strength beyond a certain level did not cause stronger contractions. He concluded that the extrinsic electric stimulus is not the direct cause of contraction, but merely serves to release the flow of intrinsic electric charges that actually activate the muscle.

Despite the solid experimental foundation and the conceptual wealth of these ideas Galvani's work remained largely ignored by the contemporary scientific community. Soon after his classical studies were completed, Galvani was stripped from his professorship for political reasons, and died destitute in 1798.

Two years later in 1800, Volta announced his invention of the electric battery [8, 9]. The great success of this discovery further diverted attention from Galvani's work, and the idea of "animal electricity" became dormant for several decades. It was resurrected by the investigations of Davy, duBois Reymond, Helmholtz, Bernstein, Overton and others, and stimulated the rich harvest of electrophysiology during the next 200 years [10a, b].

The details of the complex electrical signalling mechanism advocated by Galvani were eventually formulated by Hodgkin, Huxley and Katz in their famous equation [11a, b] that became the cornerstone of electrophysiology.

On the bicentennial celebration of Galvani's birth in 1937, Niels Bohr said [8]: "This immortal work . . . which inaugurated a new epoch in the whole field of science, is a brilliant illustration of the fruitfulness of an intimate combination of the exploration of the laws of inanimate nature with the study of the properties of living organisms."

EARLY MORPHOLOGICAL OBSERVATIONS ON SARCOPLASMIC RETICULUM

The late 19th century was an exciting period of muscle biochemistry [1, 12, 13]. The isolation and characterization of myosin by Kühne [14–16] was soon followed by the first observations on actin [17–19], a detailed description of the reticular system of muscle [20–21], later identified as the sarcoplasmic reticulum and T-tubules [22–24], and the first observations on the role of calcium in the contractility of heart and skeletal muscle [25–30]. Within 80 years after the description of calcium by Humphrey Davy in 1808, the essential elements of the contractile system were already identified and analyzed in some detail.

The first observations on the network of longitudinal and transverse membrane elements in the spaces between myofibrils were published by Gustav Retzius [20, 21]. Using the leg muscles of the water beetle and a gold impregnation technique that stained preferentially the membranous structures, Retzius described three sets of transverse networks, one at the level of the Z line, one near the middle of the sarcomeres, and a third set between them. The position of the third network corresponds to the location of the transverse tubules within the dyads [13, 31]. As Bowman [32] before him, Retzius associated the contraction process with the myofibrils, and suggested that the network of transverse elements stained by gold may be involved in the spread of excitation through the muscle fiber. This farsighted prediction soon became disputed and several notable microscopists, including Cajal, proposed that the filaments stained by gold are actually the contractile structures.

Experimental evidence for the opening of the transverse network to the extracellular space was obtained by Nystrom as early as 1897 [33]. He observed the formation of dark lines crossing the myofibrils at spacings equal to that of the striations, after injection of India ink into heart muscle. Holmgren [34] attributed these observations to the entry of India ink particles into the muscle fibers near the center of the I band, where transverse tubules were later found by local excitation and electron microscopy [35, 36]. Using the chrome-osmium staining method, Retzius was probably also the first to describe definitively elements of sarcoplasmic reticulum [31, 37].

The light microscope observations on the transverse and longitudinal reticulum culminated in the classic paper of Emilio Veratti published in 1902 [22], and reprinted in English translation in 1961 [23]. Using the black reaction of Golgi, Veratti observed a complex, delicate network of longitudinal and transverse filaments in a wide variety of muscles at different stages of development and illustrated them in beautiful drawings, that accurately represent their distribution in the longitudinal and transverse plane of the muscle fiber. This was a remarkable achievement in view of the small dimensions of the structures, that would put them at the limit of light microscopic resolution. Muscles with one, two, or three transverse reticular networks per sarcomere were seen and although these were often dismissed by other light microscopists as preparation artifacts, their existence has been fully confirmed by electron microscopy [24, 37, 38].

These remarkable observations made little impact at their time, since the physiological context that would have given them real meaning developed a half century later [35].

Instead of stimulating continued interest, Veratti's work inaugurated a lull in the structural research on muscle. As Barer wrote in 1948: "Relatively little interest seems to have been taken in the problems of muscle structure in the years between 1900 and 1930. The old observers attacked the problem with such energy and in so many ways that there seemed relatively little left to do" [31]. For about 50 years between the publication of Veratti's paper in 1902 and its rediscovery by electron microscopy, the sarcoplasmic reticulum and the transverse tubular system was nearly completely forgotten. Bennett wrote in 1960: "It is astonishing that a structure once described as accurately and as beautifully as the reticulum was by Veratti (1902) should have so quickly become almost lost to man's knowledge" [39].

With the use of the electron microscope the sarcoplasmic reticulum was found to consist of membranous tubules and cisternae forming a fine sleeve-like network around the myofibrils [37, 38]. The tubular elements are arranged in a definite pattern with respect to the sarcomere, that is repeated with the striation of myofibrils.

The transverse T-system represents the invagination of the surface membrane of muscle cell into the cell interior [40–48]. The direct continuity of the lumen of transverse tubules with the extracellular space, first suggested in 1897 by the observations of Nystrom [33], was conclusively proved half a century later by the penetration of ferritin [44, 45], fluorescent dyes [49], and serum albumin [50] deep into spaces that correspond in localization and approximate volume to the transverse tubules.

A prominent feature of the intracellular membrane system is the triad formed by two cisternal enlargements of the sarcoplasmic reticulum which lie adjacent to a T-system tubule [48, 51]. The triad serves as the transmitter of the excitatory stimulus from the surface membrane *via* the T-tubules to the sarcoplasmic reticulum, activating the release of Ca^{2+} that triggers muscle contraction [52].

Veratti [22, 23] first observed that the localization of the triads with respect to the myofibrillar structure is different in various species. In the frog sartorius [53], the tail muscles of amphibian larvae [54], and in the white muscles of some fish [42, 43], the triads are located at the level of the Z-line, while in the lizard [55, 56], rat [54], toadfish swim bladder [57], mouse [58], bat [59], and some fish eye muscles [60], they are found near the ends of the A-bands. The physiological significance of this characteristic relationship became evident with the demonstration that the localization of triads corresponded to the regions of sarcomere where contraction could be elicited by local excitation with microelectrodes [35, 61–63]. These experiments gave substance to the earlier suggestions of Retzius [20, 21] that the T-system plays a role in the conduction of the excitation into the interior of the muscle cell and opened the way for the elucidation of the molecular mechanism of excitation-contraction coupling [52, 64–66]. The requirement for such a signal conduction pathway was predicted earlier by A.V. Hill [67a, b], based on the argument that diffusion of an activating substance from the surface membrane to the center of the muscle fiber would be too slow to explain the rate of tension development.

As the lines of physiological and biochemical investigations converged, the activating substance of muscle contraction was eventually identified as calcium and the sarcoplasmic reticulum became recognized as the principal regulator of the Ca^{2+} concentration of sarcoplasm [35, 52, 63, 68–71].

CYTOPLASMIC Ca^{2+} AS A REGULATOR OF MUSCLE CONTRACTION

The observations of Sydney Ringer on the effects of extracellular Ca^{2+} on the contractility of frog heart and skeletal muscles [25–28] represent the first major step in the recognition

of Ca^{2+} as a key regulator of muscle contractility [29, 30]. Ringer found [25, 27] that when isolated frog or eel hearts are perfused with a 0.5–0.6% NaCl (saline) solution, "the contractions rapidly get weaker. . . . In about twenty minutes the heart ceases its spontaneous beats and becomes inexcitable by even strong induction shocks."

"Potassium salts added to the circulating saline do not enable the heart to maintain contractility . . . indeed under their influence the ventricle ceases to contract sooner."

By contrast, "Ca salts added to the saline after the ventricle has lost its contractility restored good spontaneous beats which continued for a long time." Ringer further observed that in saline-calcium solutions "the beats eventually became broader, the diastolic dilatation was delayed and fusion of the beats led to a state of tetanus." These secondary effects of calcium could be prevented by the addition of potassium in physiological concentrations. Ringer concluded the extracellular Ca^{2+} is required to preserve the contractility of the heart but calcium, potassium, and sodium must be present in correct proportions (as in the Ringer's solution) for normal heart activity.

The rhythmic contractions of smooth muscle in frog esophagus strips showed similar dependence on extracellular Ca^{2+} and potassium, as the frog heart [72].

In contrast to heart and smooth muscles, the contractility of frog skeletal muscles can be maintained for several days in the absence of added Ca^{2+} in the extracellular fluid [26, 28, 73]. Even under continuous electric stimulation at 30/min frequency, the contractility of skeletal muscles persisted in Ca^{2+}-"free" solutions for several hours. Ringer further noted that in contrast to heart, high external Ca^{2+} "caused no prolongation of the beat and no delay of relaxation in skeletal muscles" [28].

To explain the striking differences in Ca^{2+} requirement between heart and skeletal muscles, Ringer considered but then rejected the possibility [28] that in frog heart there is a relatively free exchange of calcium and potassium between the cell and the environment, while in the sartorius muscle the diffusion of ions occurs much more slowly, making it relatively insensitive to the ion composition of the medium.

It became clear a half century later, that heart muscle is indeed more dependent on the influx of extracellular Ca^{2+} for excitation than skeletal muscle, and it is less able to retain its intracellular calcium store in a Ca^{2+}-poor perfusion medium [74–78].

The investigations started by Ringer were continued by Locke, Loeb, Loew, Heilbrunn and others, implicating Ca^{2+} as a regulator in a broad spectrum of biological processes in excitable and nonexcitable cells [29, 79].

There is a series of early observations that indicate the involvement of intracellular Ca^{2+} in the contraction of skeletal muscle, in spite of its insensitivity to extracellular Ca^{2+}. It became known already in the 1920's that microinjection of Ca^{2+}-precipitating anions such as phosphates, carbonates, sulfates [80], or alizarin sulfonate [81], into amoebas caused immediate cessation of movement, and movement could be restored by microinjection of a dilute solution of calcium. Chambers & Hale [82] observed that 0.1 M Ca^{2+} blown on the surface of a frog muscle fiber from a micropipette caused contraction. Reversible contraction was seen by Keil & Sichel [83] after microinjection of 5 mM $CaCl_2$ solution into single fibers of frog adductor muscle. A solution of NaCl had no such effect.

Weise [84] found that an ultrafiltrate of rat skeletal muscle dispersed in saline contained no detectable Ca^{2+}, implying sequestration of Ca^{2+} in some cellular structures; however, when the rats were previously subjected to intense treadmill exercise, about 50% of the Ca^{2+} content of the muscle appeared in the ultrafiltrate, indicating the release of a large amount of calcium from an intracellular calcium sink during contraction.

Muscle exercise or violent muscle contractions were indeed known to increase the calcium concentration of the blood [85–87], and

Woodward [88] observed an increased rate of release of radioactive calcium from frog sartorius muscles upon stimulation. These observations are all consistent with increased cytoplasmic Ca^{2+} concentration in contracting muscles.

Bailey made the remarkable prophetic prediction in 1942 [89]: "We suggest that the essential feature of excitation and contraction – we cannot at present dissociate the two phases – is the liberation of the Ca ion in the neighborhood of the ATPase grouping which can thus by the almost instantaneous catalysis of ATP breakdown make available a large amount of energy. For it is legitimate to assume that the living cell in the resting state can provide a mechanism for the separation of enzyme and activator until they are brought together as the result of excitation." While we now know that the direct activation of myosin ATPase by Ca^{2+} at millimolar concentration does not play a role in muscle contraction, the clear logic and the essential validity of Bailey's argument is impressive.

The studies of Heilbrunn [90–93], together with those of Kamada & Kinoshita [94], were clearly the most influential in focussing attention on Ca^{2+} as the trigger substance of muscle contraction [95]. Heilbrunn [90] observed that when isolated muscle fibers with cut ends were immersed into solutions containing calcium, rapid shortening ensued. Similarly, minced pieces of living frog muscle placed in dilute calcium chloride solutions showed marked loss of weight due to contraction and extrusion of fluid, an effect not duplicated by potassium, sodium, or magnesium (work of Miss M. Willis, cited by Heilbrunn & Wiercinski in [93]).

These observations formed the basis of the Ca^{2+} release theory of muscle contraction put forward by Heilbrunn in 1943, in which Ca^{2+} released from internal storage sites into the muscle cytoplasm by electrical or chemical stimuli, was assumed to activate the contractile material. The Ca^{2+} release theory gained further support by the demonstration [93, 94] that microinjection of Ringer solution containing 0.2–1.25 mM Ca^{2+} into frog muscle fibers caused rapid muscle contraction, while injection of Ca^{2+}-free Ringer solution was without effect. Isotonic (0.125 M) KCl usually caused lengthening of the fiber instead of contraction, but occasionally genuine contraction was also observed. This irregular effect of K^+ was attributed by Heilbrunn to Ca^{2+} release from the bound state induced by K^+ [91, 93]. Injection of isotonic Na^+ or Mg^{2+} was usually without effect, but microinjection of Ba^{2+}, like Ca^{2+}, produced rapid shortening [93].

At the time of these studies the prevailing view held by Szent-Györgyi [96, 97] was that K^+ is the regulator of muscle contraction. Szent-Györgyi stated: "Since Ca^{2+} and Mg^{2+} are bound strongly by myosin and are thus immobilized it will be the K^+ which will condition contraction or relaxation by its motion." The experiments of Heilbrunn & Wiercinski [93] clearly contradicted this view, and did not find ready acceptance. As we know today, the K^+ concentration of the cytoplasm remains relatively constant during contraction, and the small physiological changes in cytoplasmic $[K^+]$ have only marginal effect on the properties of the contractile material.

With the introduction of Ca^{2+}-chelators such as EGTA [98, 99], it became possible to define the precise relationship between free Ca^{2+} concentration and the state of contraction of actomyosin and myofibrils. In an elegant series of experiments using EDTA and EGTA buffer systems to control free Ca^{2+} concentration, A. Weber established that the ATPase activity and the state of contraction of natural actomyosin and myofibrils are unique functions of free Ca^{2+} concentration [100–107]. Maximum rates of ATP hydrolysis and syneresis were obtained with myofibrils in the presence of 5 mM Mg^{2+} and 1 mM ATP at a free Ca^{2+} concentration of about 10^{-5} M. At low free Ca^{2+} concentration, the actomyosin complex was dissociated by ATP with progres-

sive inhibition of both ATPase activity and syneresis, resulting in a state reminiscent of relaxation.

Parallel with these developments, Ebashi [70, 108] observed a linear relationship between the Ca^{2+} binding capacity of a series of chelating agents and their relaxing activity on glycerinated myofibrils. Thus the initially puzzling problem [109] of how the relaxing effect of EDTA and EGTA can develop in the presence of excess Mg^{2+} found a simple explanation in the preferential binding of Ca^{2+} over Mg^{2+} by the chelating agents.

In 1959 Ebashi performed the crucial experiment that established the dependence of actomyosin superprecipitation on the free Ca^{2+} concentration in the absence of chelating agents or sarcoplasmic reticulum [70, 110, 111]. Natural actomyosin was carefully washed with EDTA solutions to remove bound Ca^{2+} and then with EDTA-free solution to remove the EDTA. The superprecipitation (serving as a model of muscle contraction) was induced by ATP in a solution of 0.06 M KCl, 1 mM ATP, 0.02 M $MgCl_2$ and 0.02 M Tris/maleate buffer, pH 6.74, at free Ca^{2+} concentrations ranging between 0 and 5 μM. Already at 0.2 μM Ca^{2+} concentration the superprecipitation of actomyosin was significantly accelerated by Ca^{2+} and maximum rates were reached at 5 μM [Ca^{2+}] [110].

THE RELATIONSHIP BETWEEN CYTOPLASMIC [Ca^{2+}] AND THE CONTRACTILE STATE OF LIVING MUSCLE

Application of microdroplets of a 10^{-5} M Ca^{2+} solution to muscle fibers from which the surface membrane was removed induced contraction, indicating that the concentration of free Ca^{2+} at rest must be less than 10^{-5} M [63, 112, 113]. Portzehl *et al.* [114] injected Ca-EGTA buffer solutions adjusted to different free Ca^{2+} concentrations into the giant fibers of *Maia squinado,* and demonstrated that contraction occurs at free Ca^{2+} concentrations of 0.5–1.0 μM, in agreement with observations made on isolated myofibrils and actomyosin [107].

A calcium-sensitive bioluminescent protein, aequorin, was isolated from the medusa, *Aequorea aequorea,* which responds with light emission to 10^{-6} to 10^{-8} M Ca^{2+} [115]. Microinjection of a concentrated aequorin solution into giant single muscle fibers of the acorn barnacle, *Balanus nubilis* [116, 117], permitted the measurement of the kinetics of *in vivo* Ca^{2+} release and reabsorption by changes in the intensity of aequorin luminescence. The onset of light emission, taken as an indication of Ca^{2+} release from the sarcoplasmic reticulum into the sarcoplasm, occurred almost simultaneously with the stimulus of 20 ms duration. Peak light intensity was reached when tension was about 10% of its maximum value. The light intensity began to decrease immediately after the cessation of the stimulus, while the tension continued to rise, approaching its maximum when the light emission had virtually ceased. Calcium was released as long as the membrane depolarization was maintained and correlation between the ionized calcium concentration and contractile tension was apparent in the fact that the peak of the Ca^{2+} transient coincided with the maximum rate of tension increase. These findings of Ridgway & Ashley [116] confirmed in all essential aspects the similar observations made earlier by Jobsis & O'Connor [118] under less reproducible conditions using murexide as the Ca^{2+} indicator.

In the 25 years since these pioneering observations, a variety of fluorescent and metallochromic indicators of cytoplasmic Ca^{2+} concentration were introduced [115, 119, 120], that permitted detailed kinetic analysis of Ca^{2+} transients in intact or cut living muscle fibers and established their relationship to tension development [52, 64–66, 115, 121, 122].

THE IDENTIFICATION OF SARCOPLASMIC RETICULUM AS THE REGULATOR OF CYTOPLASMIC [Ca^{2+}]. THE DISCOVERY OF Ca^{2+}-ATPase

Much of the early information on the role of sarcoplasmic reticulum in Ca^{2+} transport arose from studies on the regulation of the activity of contractile proteins by calcium. The discovery of myosin ATP hydrolysis by Englehardt & Ljubimova [123–125] was soon followed in Albert Szent-Györgyi's laboratory by the observation of the unique properties of myosin B [126], that led to the isolation and characterization of actin by Straub [127, 128], and the demonstration of its interaction with myosin, forming actomyosin. Myosin B was recognized as the natural form of actomyosin. The contraction of actomyosin threads induced by ATP, K^+, and Mg^{2+} [129] crowned these achievements and opened the way for the rapid development of muscle biochemistry. The scope and momentum of these discoveries within a span of a few years is even more astonishing, since they took place in Hungary during the turbulent years of World War II in virtual isolation from the world community of scientists [130, 131].

As soon as the basic elements of the contractile apparatus were in place, the problem of relaxation was addressed in a brief note by Szent-Györgyi [132], that anticipates some of the later developments on the role of a "cytoplasmic factor" in the relaxation process. The following passage is quoted from this report [132]: ". . . threads of myosin B show a violent contraction if suspended in a solution containing KCl (0.05 M), $MgCl_2$ (0.001 M) and ATP (adenyltriphosphate, 0.17%). If such a contracted thread is washed out with water and suspended in a solution containing 0.25 M KCl and 0.001 M $MgCl_2$ no appreciable change is observed. If ATP is added now to the solution the thread swells up within a few minutes to its original size; it becomes transparent and similar to the uncontracted thread in all respects. If the liquid is replaced by the salt solution in which the contraction was obtained, the thread contracts again. The contraction is thus reversible and ATP is essential not only for the contraction but also for the relaxation. The thread can be brought to contraction and relaxation by the variation of the KCl concentration.

Mg^{2+} is essential for the contraction as well as for the relaxation. In absence of Mg^{2+} the contraction is sluggish and there is no relaxation at all. Only a very slight swelling is obtained as revealed by the somewhat increased transparency. At higher KCl concentrations (in presence of ATP) the thread disintegrates without much swelling.

The Mg^{2+} can be replaced by a dialysed extract of the muscle. Whether this action is due to the traces of Mg^{2+}, possibly bound by the protein, or to some other substance, cannot be stated at present."

These observations clearly established the role of ATP and Mg^{2+} both in contraction and in relaxation, but the emerging body of information suggesting the role of Ca^{2+} [80–87] was ignored by Szent-Györgyi. It is reasonable to assume that the ATP and Mg^{2+} requirement for relaxation in Szent-Györgyi's experiments [132] was due largely to Ca^{2+} sequestration by the sarcoplasmic reticulum in the crude muscle extract.

The relationship between calcium and the relaxing effect of muscle extracts was clearly demonstrated about a decade later by Marsh [133, 134]. Marsh observed that after centrifugation in the presence of Mg^{2+} and ATP, the volume of sedimented crude myofibrillar fraction remained large but addition of a small amount of Ca^{2+} resulted in sudden shrinkage. Myofibrils resuspended in 0.1 M KCl responded to the addition of 1 mM ATP with immediate shrinkage even in the absence of added Ca^{2+}. These experiments suggested to Marsh the presence of a substance in the muscle extract that is intimately involved in the

Ca^{2+}-dependent regulation of the myofibrillar volume changes. He named this substance "sarcoplasmic factor". Marsh concluded that close packing and swelling of the myofibrillar sediment corresponded to contraction and relaxation, respectively, and that the sarcoplasmic factor regulated contraction and relaxation [133, 134]. The sarcoplasmic factor also inhibited the myofibrillar ATPase activity, whose connection with muscle contraction was known since the experiments of Engelhardt & Ljubimova [123–125] and Szent-Györgyi [129]. The studies of Marsh were soon extended by Bendall [135–138] and by Hasselbach & Weber [139], who found that glycerol-extracted muscle fibers brought to the state of contraction by ATP in the presence of Mg^{2+} could be made to relax by the addition of Marsh's factor.

The relaxing activity of the muscle extract could be recovered almost completely in a sediment obtained either by differential centrifugation of the muscle extract at 8 000 to 30 000 × ***g*** or by ammonium sulfate fractionation [140–143]. The sedimentation properties and Mg^{2+}-activated ATPase activity of the relaxing factor resembled the granular ATPase described earlier by Kielley and Meyerhof in their classic papers [144–146]. Electron microscopic evidence suggested that the vesicular membrane fragments originated from the sarcoplasmic reticulum [147, 148].

Ebashi discovered that in the presence of ATP and Mg^{2+}, sarcoplasmic reticulum fragments actively removed Ca^{2+} from the medium [110, 111, 149]. The initially puzzling ATP-dependent Ca^{2+}-binding process was identified by Hasselbach as a powerful Ca^{2+} transport system which derived its energy from the hydrolysis of ATP through a Ca^{2+}-specific transport-ATPase [71, 150–152] that was capable to lower the Ca^{2+} concentration of the medium to submicromolar levels with inhibition of the ATPase activity of myofibrils. The Ca^{2+} transport ATPase was activated by μmolar Ca^{2+} in the presence of 5 mM Mg^{2+} (extra-ATPase). The Ca^{2+}-stimulated "extra" ATP hydrolysis was stoichiometrically related to the transport of Ca^{2+}, and was inhibited by the SH group blocking agent Salyrgan.

For a short while doubts were expressed about the identity of the Ca^{2+} storing membrane vesicles with the sarcoplasmic reticulum [71], since at about the same time Vasington & Murphy [153] demonstrated ATP-dependent Ca^{2+} storage in mitochondria. These doubts were soon dispelled with the demonstration of Ca^{2+}-oxalate deposits in the terminal cisternae of sarcoplasmic reticulum in sections of glycerol-treated muscle fibers after incubation with Mg^{2+}, ATP, oxalate and Ca^{2+}-EGTA [154–156], that definitely linked the Ca^{2+} transport activity with sarcoplasmic reticulum elements.

Based on the close relationship between ATP-ADP exchange and Ca^{2+} transport [149, 151], the following hypothesis was proposed to explain the ATP-dependent accumulation of Ca^{2+} by sarcoplasmic reticulum [152]: ". . . ein unbekannter Carrier in der Membran vorhanden ist, der auf der Aussenseite der Membran durch ATP phosphoryliert und auf der Innenseite dephosphoryliert wird. Ein solcher Carrier erklart alle Beobachtungen, wenn angenommen wird, dass er im phosphorylierten Zustand ein Calciumion mit einer Affinitat bindet, die 500 mal grosser ist als die Affinitat im nichtphosphorylierten Zustand, und ein zweites Calciumion mit einer Affinitat, die nur 150 mal grosser ist als die Affinitat des nichtphosphorylierten Carriers. Nach diesem Konzept wird von einem ATP immer nur ein Carrier phosphoryliert, aber dieser phosphorylierte Carrier transportiert zwei Calciumionen."

The discovery of the Na^+, K^+-transport ATPase paralleled these developments [157–162]. The formation of an aspartylphosphate enzyme intermediate (E∼P) became recognized as the central feature of the mechanism of the whole class of so-called P-type ion trans-

port ATPases, that include the Ca^{2+}-ATPases of sarcoplasmic reticulum, the Ca^{2+}-ATPases of surface membrane, the Na^+, K^+-ATPase and the H^+, K^+-ATPase of gastric mucosa [163, 164].

The relaxing effect of skeletal muscle sarcoplasmic reticulum vesicles is satisfactorily explained by their ability to accumulate Ca^{2+}. Inhibition of the syneresis and ATPase activity of actomyosin and myofibrils by EGTA or by sarcoplasmic reticulum fragments was accompanied by a reduction of their bound Ca^{2+} content [100–107]. Mueller [165] further found that sarcoplasmic reticulum vesicles not only inhibited the shrinkage of actomyosin precipitates by ATP, but also reversed the ATP-induced volume change and promoted the solubilization of actomyosin at low ionic strength. All these effects of the relaxing factor were counteracted by calcium.

In turn, in the presence of Mg^{2+} and ATP, EDTA and other chelating agents caused the relaxation of contracted glycerinated fibers [137, 166–168], the clearing of actomyosin suspensions, and inhibited the ATPase activity of myofibrils, although EDTA did not bind to actomyosin [169].

The changes in myofibrillar activities and in the amount of Ca^{2+} bound to myofibrils depend on the free Ca^{2+} concentration in a similar manner whether they resulted from Ca^{2+} binding by chelating agents or by skeletal muscle microsomes [106, 107].

Oxalate, a potent activator of the relaxing effect of skeletal muscle microsomes, augmented the uptake of Ca^{2+} by serving as a Ca^{2+}-precipitating agent in the interior of microsomal vesicles [170, 171]. Potentiation of Ca^{2+} transport and, in some cases, relaxing activity, were also observed with pyrophosphate, inorganic phosphate, and fluoride [172]. All these anions are characterized by the low solubility of their Ca^{2+} salts suggesting that, similarly to oxalate, they also serve as Ca^{2+}-precipitating agents.

Two obstacles delayed the acceptance of the idea that Ca^{2+} regulates muscle contraction by direct interaction with the contractile proteins:

1. Alternative mechanisms of the relaxing effect of sarcoplasmic reticulum advocating the formation of "soluble relaxing factors" and the participation of ATP rephosphorylating systems such as myokinase, ATP : creatine phosphotransferase and pyruvate kinase were in circulation, and coexisted for a while with the Ca^{2+} mechanism.

Doubts soon began to emerge about these elusive effects and within a few years the "soluble relaxing factor" turned out to be simply the effect of an incubation medium rendered essentially calcium-free by the Ca^{2+} transporting activity of sarcoplasmic reticulum during the preincubation with sarcoplasmic reticulum vesicles [173].

As ATP rephosphorylating enzymes had no influence on the relaxing activity of sarcoplasmic reticulum under conditions where the ATP concentration was maintained at saturating level, the effect of ATP-rephosphorylating enzymes could be explained by the removal of ADP from the reaction systems [174].

2. The second problem was that the contractile activity of actomyosin prepared from pure actin and pure myosin was unaffected by chelating agents and generally insensitive to calcium [175], raising doubts about a direct effect of Ca^{2+} upon actomyosin. This problem was soon resolved by the discovery of the thin filament protein, troponin [176, 177], that binds the activating Ca^{2+} and triggers contraction by a cascade of conformational changes that modulate the interaction of actin and myosin [69, 70, 178, 179a, b].

The information outlined above clearly established that the contractile system is regulated by the concentration of free cytoplasmic Ca^{2+} and that sarcoplasmic reticulum is able to lower the free Ca^{2+} concentration to the level one would expect to find in relaxed muscle. Using this information, a hypothetical picture of events occurring during a contraction-relaxation cycle was drawn, based on the participation of sarcoplasmic reticulum [63].

In the relaxed state Ca^{2+} is stored in the sarcoplasmic reticulum and the concentration of free Ca^{2+} in the sarcoplasm is low (about 10^{-8} M). On excitation, the depolarization wave generated by the nerve impulse spreads through the T-system into the interior of the muscle fiber and triggers the release of Ca^{2+} from the sarcoplasmic reticulum into the sarcoplasm. The elevation of the Ca^{2+} concentration in the environment of the myofilaments brings about the contraction by interaction with troponin. As the membrane is repolarized, the concentration of Ca^{2+} in the sarcoplasm is lowered by the sarcoplasmic reticulum, troponin releases its bound calcium, actomyosin is dissociated, and relaxation ensues.

The relative abundance of sarcoplasmic reticulum membranes in different muscles was found to be roughly proportional to their rate of contraction and relaxation [180]. At the fast end of this spectrum is the cricothyroid muscle of the bat, which produces the bursts of frequency-modulated sound used by these animals for echolocation. The extensively developed sarcoplasmic reticulum of this muscle is related to this unusual physiological demand [59]. A similar example is the swim bladder muscle of the toadfish [57], that produces the deep, resonant sound of this species, by high-frequency contraction and relaxation.

In line with the early observations of Veratti [22, 23], the sarcoplasmic reticulum is still weakly developed in embryonic muscles [181]. The Ca^{2+}-ATPase content of muscle rapidly rises around birth [181, 182], parallel with the increase in contractile activity, suggesting coordinated expression of the contractile proteins and the sarcoplasmic reticulum Ca^{2+}-ATPase [183, 184].

The Ca^{2+}-ATPase is a major component of sarcoplasmic reticulum representing close to 80% of its protein content in fast-twitch skeletal muscle [163, 185]. Due to its great abundance, a few cycles of Ca^{2+} transport are sufficient to bring about relaxation.

The principal regulator of the activity of Ca^{2+}-ATPase is the Ca^{2+} concentration in the cytoplasm and in the lumen of the sarcoplasmic reticulum [186, 187], but in cardiac muscle phospholamban also contributes to the regulation of Ca^{2+} transport [188]. In the relaxed muscle the Ca^{2+}-ATPase is near equilibrium and the rate of ATP hydrolysis is slow.

The determination of the amino acid sequence of Ca^{2+}-ATPase [189] opened a new era in the analysis of ion transport mechanism. Three major families of Ca^{2+}-ATPases (SERCA 1-3) were identified, representing the principal isoforms of fast-twitch skeletal muscle (SERCA 1), slow-twitch skeletal and cardiac muscles (SERCA 2), and smooth muscle or non-muscle cells (SERCA 3); within each family several isoforms may be produced by alternative splicing [163, 187]. The small but significant functional differences between the various isoforms contribute to the differences in Ca^{2+} regulation between muscles of distinct fiber types [180].

The crystallization of Ca^{2+}-ATPase in several distinct crystal forms [185, 190a, b–192], together with structure predictions from the amino-acid sequence, identified the major structural domains of the enzyme, but further work at atomic resolution is required for precise correlation of structure with Ca^{2+} transport function.

THE MECHANISM OF Ca^{2+} REGULATION AND THE DISCOVERY OF TROPONIN

The faint outline of muscle structure revealed by Leeuwenhoek in 1674 [1–3] became refined by electron microscopy and X-ray diffraction to molecular dimensions three centuries later [193a, b]. Interdigitating sets of thick myosin-containing filaments and thin actin- containing filaments were identified as the principal constituents of the A and I bands of the sarcomere, respectively. The recognition of the joint role of actin and myosin in

tension generation led to the hypothesis that interaction of myosin and actin during ATP hydrolysis is the basis of muscle contraction and their dissociation by ATP is the process underlying relaxation [194]. The interaction between actin and myosin occurs through myosin cross-bridges projecting at definite intervals from the thick filaments. The shortening of muscle fibers arises from the sliding motion of thin filaments relative to the thick filaments driven by the cross-bridges [193a, b, 194]. The cross-bridges formed by the globular heads of myosin molecules contain the ATP and actin binding sites and undergo cyclic structural changes during ATP hydrolysis [195–199].

Intensive search for myofibrillar proteins other than actin and myosin began after Perry & Grey [175] made the surprising observation that in contrast to the large Ca^{2+}-dependent changes in the ATPase activity and contractile state of myofibrils, glycerinated muscle fibers and natural actomyosin (myosin B), the ATPase activity of reconstituted actomyosin made from pure myosin and pure actin was not affected by EDTA. This implied either that some regulatory component of the actomyosin system was lost during purification, or that myosin or actin underwent some changes during isolation, that caused the loss of Ca^{2+} sensitivity. Weber & Winicur [102] found that some reconstituted actomyosin preparations retained their sensitivity to EGTA and this property was always associated with the actin rather than with the myosin component used for reconstitution.

The enigma was eventually solved by Ebashi with the isolation of a new myofibrillar Ca^{2+} binding protein, first named native tropomyosin [176, 177], and later renamed troponin [70, 178, 179]. The loss of this protein during the isolation of actin explained the earlier observed insensitivity of reconstituted actomyosin to calcium, [102, 175]. The troponin complex contains three components: troponin C interacts with Ca^{2+}, troponin T binds to tropomyosin, and the troponin I or inhibitory component binds to actin [70]. The association of troponin and tropomyosin with actin in the thin muscle filaments imparts Ca^{2+} sensitivity upon the ATPase activity of actomyosin and regulates the contractile state of the muscle in response to changes in cytoplasmic [Ca^{2+}].

The molecular mechanism of Ca^{2+} regulation involves a complex interplay between Ca^{2+}, Mg^{2+}, ATP, actin, tropomyosin, myosin and the three components of troponin [70, 200–202]. In the relaxed muscle the cytoplasmic Ca^{2+} concentration is low and troponin is essentially Ca^{2+}-free; under these conditions the tropomyosin molecules situated in the groove of the actin helix block the interaction of actin and myosin and maintain a relaxed state even in the presence of ATP [193a, b, 203]. During muscle activation the cytoplasmic Ca^{2+} concentration rises. Ca^{2+} binding to troponin C changes its conformation [204, 205] moving tropomyosin out of its blocking position [193a, b, 203] permitting the attachment of myosin cross-bridges to actin and tension develops. This thin-filament based Ca^{2+} regulation, with troponin as Ca^{2+} sensor, is dominant in mammalian skeletal muscles.

The study of scallop muscles [206, 207] and various mammalian smooth muscles [208, 209] revealed interesting variations on the theme of Ca^{2+} regulation. In scallop muscles instead of a thin-filament based Ca^{2+} regulation, Ca^{2+} binding to myosin light chains activates the contraction [206, 207]. In most smooth muscles Ca^{2+}-dependent phosphorylation of myosin light chains plays a major role in Ca^{2+} regulation [208, 209]. However, smooth muscles contain tropomyosin, and a unique thin filament based regulation involving caldesmon or calponin as Ca^{2+} sensors instead of troponin, was observed [210]. The various regulatory mechanisms probably emerged independently during evolution and frequently overlap [211].

The identification of troponin as the Ca^{2+}-sensitive component of the contractile apparatus in skeletal muscle was soon fol-

lowed by the discovery of the regulation of phosphorylase *b* kinase activity by calcium ions [212] that led to the discovery of calmodulin [213, 214] and to the explosive progress in the analysis of the messenger role of Ca^{2+} and the mechanism of action of muscle and non-muscle calcium binding proteins [215, 216].

As Loewenstein wrote [217]: "Calcium has many virtues that make it quite unique among cations in its ability to complex with biological structures. Its divalency allows for a wide range of binding constants with biomolecules, its radius is compatible with peptide chelation, and its charge-to-size ratio permits it to slip into small molecular holes. Its crystal-field requirements are quite flexible, bond distances and angles are adjustable, and coordination numbers can vary from six to ten. All this gives the ion a great advantage in binding to irregular geometries of coordination sites of biological molecules that can accept the ion rapidly and sequentially and fold around it, permitting graded structural modulation. Small wonder that such an engaging character has been awarded role after role in the evolution of biological signaling!"

THE COUPLING OF EXCITATION TO CONTRACTION

The depolarization of the plasma membrane initiated by the nerve impulse is conducted into the muscle interior through the transverse tubules, reaching the triad junction [35, 63, 218]. Two components of the triad are involved in the conversion of membrane potential changes into cytoplasmic Ca^{2+} signals. The dihydropyridine receptor Ca^{2+} channel (DHPR) located in the transverse tubule serves as voltage sensor, responding with a charge movement and some Ca^{2+} influx to the change in membrane potential [52, 65, 219]. The voltage-dependent changes in DHPR activate the ryanodine receptor Ca^{2+} channel (RyR) of the sarcoplasmic reticulum, releasing Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm [66, 122]. The released Ca^{2+} activates contraction by interaction with troponin [70], and serves as a signal for the regulation of various metabolic pathways (phosphorylase kinase, pyruvate dehydrogenase, etc.) that contribute to ATP synthesis.

As the stimulus ends, the plasma membrane and the T tubules are repolarized, the DHPR returns to its resting conformation and the RyR Ca^{2+}-channels close. The cytoplasmic Ca^{2+} activates the Ca^{2+}-transport ATPase, and Ca^{2+} is transported back from the cytoplasm into the lumen of sarcoplasmic reticulum lowering the cytoplasmic $[Ca^{2+}]$ to resting levels of about 10^{-8} M. Troponin releases its bound Ca^{2+} and the muscle is relaxed.

During the last two decades the amino-acid sequences of DHPR [220] and RyR [221] have been established and the three dimensional structure of RyR was defined at moderate resolution by electron microscopy [222, 223]. Progress was also made in identifying some of the regulatory mechanisms that control the changes in cytoplasmic Ca^{2+} concentration in skeletal [52, 66] and cardiac [224] muscles. Unraveling the molecular details of the Ca^{2+} release process will require higher resolution structures of DHPR and RyR than currently available.

The emerging molecular mechanism of excitation-contraction coupling is a fitting tribute for the bicentennial of Galvani's accomplishments.

Ca^{2+} AND GENE EXPRESSION

Compared with our understanding of excitation-contraction coupling and the structural basis of force generation, relatively little is known about the genetic regulatory mechanisms that control the coordinated expression of muscle proteins during development [184, 225–227] and their adaptation to functional demands in adult animals [180, 228–230]. Myogenic, neural, and hormonal mechanisms

modulate these processes through signaling cascades in the cytoplasm and nuclei, that include Ca^{2+} as a key messenger [184, 231–233]. Major expansion of our knowledge in these areas is required to answer the many open questions related to muscle differentiation, exercise, aging, and muscle diseases.

CONCLUDING REMARKS

This brief review of the history of muscle research reaffirms the opinion of A.F. Huxley [13]: "Biologists of all kinds owe a tremendous debt to their predecessors of around a century ago — some of course owe more than others, but the differences are more in their readiness to acknowledge the debt than in the size of the debt itself."

This observation also applies to our times. The avalanche of new information rapidly buries yesterday's results, creating a forgotten literature in which treasures may remain hidden, simply because we do not have the time or interest to find them. Siekevitz wrote [234]:

"There is much out there which has been and is disregarded as not worthy of even being sought out, perhaps because it is published in journals not of self-acclaimed first rank, from laboratories little known, from countries ill-regarded."

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