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Calcium ion and troponin: Professor S. Ebashi's epoch-making achievement

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Abstract

The processes by which Professor Setsuro Ebashi accomplished his great work are described. Independently of Marsh, Ebashi discovered the relaxing factor in homogenized muscle and showed that it has a lipid-containing particulate fraction with ATPase activity, later identified as the sarcoplasmic reticulum. He then solved the mechanism of relaxation of the relaxing factor through the following findings. A minute amount of calcium ion (Ca^{2+}) is necessary for the physiological contractile reaction. The relaxing factor strongly accumulates Ca^{2+} in the presence of ATP and sufficiently removes Ca^{2+} from the contractile system to bring about relaxation. Ebashi found that the contractile reaction of myosin and actin is regulated by Ca^{2+} only in the presence of a tropomyosin-like protein factor, which he later showed to be a complex of tropomyosin and a new protein, troponin. He proved that troponin is the Ca^{2+}-receptive protein and proposed the correct scheme for the molecular mechanism of regulation of contraction and relaxation.

Keywords: Calcium ion; Relaxing factor; Troponin; Sarcoplasmic reticulum; Glycerinated fiber; Excitation–contraction coupling

Ca^{2+} is well known to play an essential role in cellular functions as a major intracellular messenger, but much less well known is that Professor Setsuro Ebashi is the one who initiated the calcium era. In the 1950s and 1960s, Ebashi explored the mystery of the contraction–relaxation cycle of striated muscle and disclosed the essential role of Ca^{2+} and its molecular mechanism of action with the crucial discovery of a Ca^{2+}-receptive protein, troponin. The steps leading to his great achievement, however, were not steady, but rather staggering, and therefore are instructive to every scientist. I describe these steps in this article, as I had a chance to witness them closely as one of his pupils.

In all the papers written by Ebashi and his coauthor(s) cited in this article, Ebashi was always the author who contributed the most. Therefore, for the sake of simplicity in this article, when Ebashi's work done in collaboration with his colleague(s) is described, the names of the colleagues are not mentioned. I am sure that all his collaborators will understand.

The independent discovery of the relaxing factor

Having been impressed greatly by the book *Chemistry of Muscular Contraction* by Szent-Gyorgyi [1], Ebashi changed his subject in spring 1952 from choline acetylase, on which he had also done some nice work [2], to muscle. He began to enjoy confirming ATP-induced contraction of glycerinated muscle fibers described in the book, but soon realized that the contracted fibers did not relax even after ATP was washed out. This was quite different from drug-induced contractions of living muscle, which quickly relaxed upon washout of the agent. Unless the contracted fibers could somehow relax again, the "contraction" might not be physiological but mere degeneration, he thought. Then his friend Dr. S. Takagi told him about Bozler's paper showing that high concentrations of ATP could

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reverse the contraction of glycerinated fiber [3]. However, the concentration of ATP required for the relaxation was too high to be physiological, and Ebashi hypothesized that ADP or some other possible impurities might hinder the relaxation. He tried to prepare ultra-pure ATP from rabbit muscle by himself. Although slight improvement was obtained, the concentration of ATP required for relaxation was still far higher than physiological. After a 3-month struggle, he noticed that newly prepared glycerinated fibers relaxed easily, but fibers kept for a long time in glycerol firmly resisted relaxing. This suggested to him that in living muscle there must be something necessary for relaxation (relaxing factor) that degenerates during storage of glycerinated fibers. He homogenized frog muscle, centrifuged it at 6000 rpm, and added the supernatant together with ATP to contracted glycerinated fibers. Beautiful relaxation was obtained! Toward the end of 1952, he reported the result at a Muscle Meeting in Japan organized by Professor R. Natori.

The essential principle of the relaxing factor

In early 1953, Professor H. Kumagai, Ebashi's mentor, told Ebashi about Bendall's paper that reported very similar work [4]. Reading the paper, Ebashi knew that the relaxing factor had been discovered by Marsh and already reported in 1951 [5]. This was not a disappointment to young Ebashi, however, but rather an encouragement because it proved that the direction of his research was right. He then inquired into the entity of the relaxing factor by ammonium fractionation and found that two fractions were necessary for complete relaxation of glycerinated fibers that had been preserved for a long period. One was ATP-regenerating enzymes, myokinase and/or creatine phosphokinase, and the other, the essential one, contained a considerable amount of lipid and had strong ATPase activity thought to be the same as Kielley–Myerhof's MgATPase [6]. This finding was reported in *Nature* in 1955 [7]. At that time most scientists working in this field believed that the essential principle of the relaxing factor was the ATP-regenerating enzymes, because these scientists worked on rather newly prepared preparations in which Kielley–Myerhof's ATPase was still alive. Ebashi's success was apparently brought about because he knew that the relaxing factor loses its function upon aging in glycerol.

Mechanism of relaxation by the relaxing factor

The next problem was the mechanism by which the relaxing factor causes relaxation. Bozler had already reported that Ca^{2+} reverses relaxation induced by a high concentration of ATP [8]. He further showed that EDTA causes relaxation of contracted glycerinated fibers in the presence of excess Mg^{2+}, which suggested that chelation of Ca^{2+} by EDTA is the cause of relaxation [9]. At that time biochemists working on muscle contraction did not notice the importance of Ca^{2+} because Ca^{2+} apparently exerted no effects on the myosin–actin–ATP interaction, the in vitro contractile response. This was because, while concentrations of Ca^{2+} that profoundly affect the contractile reactions are on the order of only micromolar as Ebashi later demonstrated, such a low concentration of Ca^{2+} was constantly present in all the experimental solutions as a contamination from the glass and chemicals of those days. However, Ebashi thought that removal of Ca^{2+} may be the cause of relaxation and that the relaxing factor, like EDTA, might remove Ca^{2+} from the medium. To examine this hypothesis, he collected various chelating agents and compared their Ca^{2+}-binding activity with their relaxing activity. To his disappointment, he found no correlation between the two activities [10]. He also examined whether Ca^{2+} is bound to the relaxing factor, but the result was negative too.

Deeply disappointed, he joined Lipmann's laboratory at the Rockefeller Institute at the end of 1958, as Kumagai had arranged. He was inclined to change his subject to enzymology, but Lipmann properly advised him to continue his own muscle research, which Ebashi gratefully acknowledged later. In early 1959, he realized that in his calculation of the Ca^{2+}-binding activity of chelating agents in Japan, he did not take their Mg^{2+} binding into account. Corrected calculations showed striking parallelism between the Ca^{2+}-binding activity and the relaxing activity of all the chelating agents used [11]. At this point Ebashi was firmly convinced that Ca^{2+} was the key substance, and he reexamined whether the relaxing factor bound Ca^{2+}. He also realized that former experiments with negative results were performed without addition of ATP, whereas all the relaxation experiments on glycerinated fibers were conducted in the presence of ATP. Thus, he found that the relaxing factor strongly accumulated Ca^{2+} in the presence of ATP, but lost the activity upon aging [12]. Electron microscopic examination of the relaxing factor (made by Palade) showed that it is fragmented sarcoplasmic reticulum and that membranes of vesicles in aged preparations were ruptured [12].

Among electron micrographs of the relaxing factor, even a triad was seen [12]. At that time, a triad drew the attention of physiologists as the site of excitation–contraction coupling. Because contraction is the reverse of relaxation, Ebashi correctly postulated at this point (and published a little later [13]) that excitation somehow causes release of Ca^{2+} from the sarcoplasmic reticulum to cause contraction; the sarcoplasmic reticulum reaccumulates Ca^{2+} when action potentials cease, and relaxation follows.

If the above picture is true, the myosin–actin–ATP interaction should be regulated by Ca^{2+}. Ebashi made a vigorous effort to remove contaminated Ca^{2+} from actomyosin preparations completely and succeeded in demonstrating that a minute amount of Ca^{2+}, on the order of micromolar, is necessary for ATP-induced superprecipitation of actomyosin [14]. At about the same time, Weber also published the result that Ca^{2+} is required for actomyosin ATPase activity [15].

Criticism of the Ca^{2+} theory

Despite Ebashi's convincing pieces of evidence as well as Weber's described above, the Ca^{2+} theory was not readily accepted by the community of muscle biochemists. One reason was that biochemists at that time did not like Ca^{2+}. Their general thought appeared to be that such an important physiological function as the control of the contraction–relaxation cycle should be regulated by some sophisticated organic substance, not by such simple inorganic matter as Ca^{2+}. It was shortly after the discovery of cAMP, and many people believed that the relaxing factor produced an organic soluble relaxing factor, which, unlike the relaxing factor itself, was able to reach actomyosin to induce relaxation. At a Muscle Conference held in 1962 at Dedham, the Ca^{2+} theory was sharply rejected.

Lipmann also did not like Ca^{2+}, and therefore it took a long time for him to accept Ebashi' results and publication of Ebashi–Lipmann's paper [12] was delayed for about 2 years. In the meantime, Hasselbach and Makinose also published results indicating that relaxing factor accumulates Ca^{2+} in the presence of ATP [16].

Another reason many researchers did not believe the importance of Ca^{2+} was the fact that actomyosin was constituted of highly purified actin and myosin, in contrast to actomyosin extracted as a complex, contracted by ATP even in the complete absence of Ca^{2+}. They erroneously thought that if Ca^{2+} were physiologically important, it would act on the highly purified contractile protein system in particular.

Discovery of troponin

Ebashi's belief, however, was not shaken. Weber had already demonstrated that Ca^{2+} sensitivity of actomyosin constituted of purified actin and myosin depends on the actin preparation; with some actin preparations actomyosin was Ca^{2+}-sensitive and with others it was insensitive [17]. Ebashi thought that Ca^{2+}-insensitive actin might be denatured. He determined to purify "genuine native" actin that would always be Ca^{2+}-sensitive. He declared his intention to carry out the project to Weber as well as to Oosawa, who was the leader of the only group working on actin in Japan, to be fair to them.

Immediately after returning to Tokyo from the Dedham Conference, Ebashi started working very hard on the project. Soon he found that the secret was not actin itself but the presence of some protein factor other than myosin and actin that confers Ca^{2+} sensitivity to the purified myosin–actin system [18]. The protein factor showed many characteristics similar to those of tropomyosin, but tropomyosin prepared by Bailey's original method [19] did not confer Ca^{2+} sensitivity [18].

Although Ebashi named the protein factor "native tropomyosin," he thought this time that the factor might be a complex of pure tropomyosin and some unknown protein. He succeeded in isolating a new protein that promoted aggregation of tropomyosin [20]. After technical improvements showed that the protein also possessed the ability to confer, in collaboration with tropomyosin, Ca^{2+} sensitivity to purified actomyosin, Ebashi named the protein troponin [21,22]. Neither troponin nor tropomyosin alone endows purified actomyosin with Ca^{2+} sensitivity, but the complex of the two proteins does.

Troponin as the Ca^{2+} receptor in myofibrils

"Native tropomyosin" in myofibrils was found to be localized along the entire thin filament by using fluorescent dye-labeled protein [23]. Soon after, by using antibody against troponin, Ebashi found that troponin molecules were regularly distributed on each thin filament with a periodicity of 40 nm [24]. The stoichiometry of actin, tropomyosin, and troponin was later shown to be 7:1:1 [25].

Troponin was found to bind Ca^{2+} strongly, while tropomyosin did not [21]. However, this did not necessarily mean that troponin was the Ca^{2+} receptor in myofibrils. It is conceivable that Ca^{2+} might bind, for example, to myosin, only at a crucial stage in the ATP-induced interaction of myosin and actin decorated with tropomyosin and troponin to regulate the reaction. Ebashi solved this problem as follows.

Ca^{2+} can be replaced by Sr^{2+} in various biological functions, which is also true for regulation of the contractile system. The contractile system of cardiac muscle has a much higher sensitivity to Sr^{2+}, more than five times greater than that of fast skeletal muscle fibers. Ebashi prepared four myofibrillar proteins—myosin, actin, tropomyosin, and troponin—separately from both cardiac and skeletal muscles and examined which protein conferred the higher sensitivity to Sr^{2+}. He found that the magnitude of sensitivity to Sr^{2+} is determined solely by the origin of troponin; i.e., if troponin was prepared from cardiac muscle, high Sr^{2+} sensitivity was obtained irrespective of the origin of the other three proteins, and vice versa [26]. Thus, troponin was proved to be the Ca^{2+}-receptive protein in the contractile system.

With these results described above, Ebashi proposed the following idea of how the troponin molecule together with Ca^{2+} regulates the interaction of myosin and actin. Upon removal of Ca^{2+} from troponin, some conformational change in the troponin molecule might be induced, and thereby inhibit the interaction of closely situated actin with myosin. Because troponin molecules are located at least 40 nm apart, they cannot exert an influence on actin molecules situated at some distance. It is more likely that the conformational change in the troponin molecule induces, through tropomyosin, a structural change in the actin filament that modifies its interaction with the myosin filament [27].

Toward the end of the 1960s Ebashi wrote two reviews [25,27], which were widely accepted. Thus, the theory of

Ca^{2+} regulation of the contraction–relaxation cycle was established.

References

[1] A. Szent-Gyorgyi, Chemistry of Muscular Contraction, second ed., Academic Press, New York, 1951.
[2] H. Kumagai, S. Ebashi, Highly purified choline acetylase, Nature 173 (1954) 871–874.
[3] E. Bozler, Mechanism of relaxation in extracted muscle fibers, Am. J. Physiol. 167 (1951) 276–283.
[4] J.R. Bendall, Effect of the 'Marsh Factor' on the shortening of muscle fibre models in the presence of adenosine triphosphate, Nature 170 (1952) 1058–1060.
[5] B.B. Marsh, A factor modifying muscle fibre syneresis, Nature 167 (1951) 1065–1066.
[6] W.W. Kielley, O. Meyerhof, A new magnesium-activated adenosine-triphosphatase from muscle, J. Biol. Chem. 174 (1948) 387–388.
[7] H. Kumagai, S. Ebashi, F. Takeda, Essential relaxing factor in muscle other than myokinase and creatine phosphokinase, Nature 176 (1955) 166.
[8] E. Bozler, Evidence for an ATP–actomyosin complex in relaxed muscle and its response to calcium ion, Am. J. Physiol. 168 (1952) 760–765.
[9] E. Bozler, Relaxation in extracted muscle fibers, J. Gen. Physiol. 38 (1954) 149–159.
[10] S. Ebashi, F. Ebashi, Y. Fujie, The effect of EDTA and its analogues on glycerinated muscle fibers and myosin adenosinetriphosphatase, J. Biochem. 47 (1960) 54–59.
[11] S. Ebashi, Calcium binding and relaxation in the actomyosin system, J. Biochem. 48 (1960) 150–151.
[12] S. Ebashi, F. Lipmann, Adenosine-triphosphate linked concentration of calcium ions in a particulate fraction of rabbit muscle, J. Cell Biol. 14 (1962) 389–400.
[13] S. Ebashi, The role of "relaxing factor" in contraction relaxation cycle of muscle, Progr. Theor. Phys. (Kyoto) (Suppl. 17) (1961) 35–40.
[14] S. Ebsahi, Calcium binding activity of vesicular relaxing factor, J. Biochem. 50 (1961) 236–244.
[15] A. Weber, On the role of calcium in the activity of adenosine 5′-triphosphate hydrolysis by actomyosin, J. Biol. Chem. 234 (1959) 2764–2769.
[16] W. Hasselbach, M. Makinose, Die Calciumpumpe der 'Erschlaffungs-grana' des Muskels und ihre Abhängigkeit von der ATP-spaltung, Biochem. Z. 339 (1961) 94–111.
[17] A. Weber, S. Winicur, The role of calcium in the superprecipitation of actomyosin, J. Biol. Chem. 236 (1961) 3198–3202.
[18] S. Ebashi, Third component participating in the superprecipitation of 'natural actomyosin', Nature 200 (1963) 1010.
[19] K. Bailey, Tropomyosin: a new asymmetric protein component of muscle, Nature 157 (1946) 368–369.
[20] S. Ebashi, A. Kodama, A new protein factor promoting aggregation of tropomyosin, J. Biochem. 59 (1965) 425–426.
[21] S. Ebashi, A. Kodama, Native tropomyosin-like action of troponin on trypsin treated myosin B, J. Biochem. 60 (1966) 733–734.
[22] S. Ebashi, A. Kodama, F. Ebashi, Troponin I: preparation and physiological function, J. Biochem. 64 (1968) 465–477.
[23] M. Endo, Y. Nonomura, T. Masaki, I. Ohtsuki, S. Ebashi, Localization of native tropomyosin in relation of striation patterns, J. Biochem. 60 (1966) 605–608.
[24] I. Ohtsuki, T. Masaki, Y. Nonomura, S. Ebashi, Periodic distribution of troponin along the thin filament, J. Biochem. 61 (1967) 817–819.
[25] S. Ebashi, M. Endo, I. Ohtsuki, Control of muscle contraction, Q. Rev. Biophys. 2 (1969) 351–384.
[26] S. Ebashi, F. Ebashi, A. Kodama, Troponin as the Ca^{++}-receptive protein in the contractile system, J. Biochem. 62 (1967) 137–138.
[27] S. Ebashi, M. Endo, Calcium and muscle contraction, Progr. Biophys. Mol. Biol. 18 (1968) 123–183.