Review

Regulation of phosphorylase kinase by low concentrations of Ca ions upon muscle contraction: the connection between metabolism and muscle contraction and the connection between muscle physiology and Ca-dependent signal transduction

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Abstract: It had long been one of the crucial questions in muscle physiology how glycogenolysis is regulated in connection with muscle contraction, when we found the answer to this question in the last half of the 1960s. By that time, the two principal currents of muscle physiology, namely, the metabolic flow starting from glycogen and the mechanisms of muscle contraction, had already been clarified at the molecular level thanks to our senior researchers. Thus, the final question we had to answer was how to connect these two currents. We found that low concentrations of Ca ions (10^{-7}–10^{-4} M) released from the sarcoplasmic reticulum for the regulation of muscle contraction simultaneously reversibly activate phosphorylase kinase, the enzyme regulating glycogenolysis. Moreover, we found that adenosine 3′,5′-monophosphate (cyclic AMP), which is already known to activate muscle phosphorylase kinase, is not effective in the absence of such concentrations of Ca ions. Thus, cyclic AMP is not effective by itself alone and only modifies the activation process in the presence of Ca ions (at that time, cyclic AMP-dependent protein kinase had not yet been identified). After a while, it turned out that our works have not only provided the solution to the above problem on muscle physiology, but have also been considered as the first report of Ca-dependent protein phosphorylation, which is one of the central problems in current cell biology. Phosphorylase kinase is the first protein kinase to phosphorylate a protein resulting in the change in the function of the phosphorylated protein, as shown by Krebs and Fischer. Our works further showed that this protein kinase is regulated in a Ca-dependent manner. Accordingly, our works introduced the concept of low concentrations of Ca ions, which were first identified as the regulatory substance of muscle contraction, to the vast field of Ca biology including signal transduction.

Keywords: low concentrations of Ca ions, phosphorylase kinase, cyclic AMP, glycogenolysis, muscle contraction, Ca-dependent protein kinase, signal transduction

1. Introduction

When I started my doctoral thesis at the end of 1965, there still remained a fundamental question on muscle physiology to be solved, albeit one with a long history of studies on it (Table 1); that is, how the breakdown of glycogen is regulated when muscles contract. Contraction is the most important and almost the sole function of muscles. It involves the transfer of large amounts of energy contained in adenosine triphosphate (ATP) present in muscle fibers after converting chemical energy into physical energy, namely, force and heat, to extramuscular organs such as bones for movement and blood for raising temperature.

Furthermore, if ATP is depleted during muscle contraction, contracture occurs, namely, the muscle cannot relax. Thus, ATP concentration must always be kept nearly constant despite ATP consumption

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Table 1. Main discoveries in muscular physiology related to this review

	Current 1: Metabolism	**Current 2: Muscular Contraction**
19C-20C	Glycogen breakdown & lactate production on muscular contraction	
1910-20	Stoichiometry of glycogen breakdown & lactate production	
	Discovery of ATP and creatine phosphate	
1930s	Discovery of creatine kinase and of the concept of phosphate group transfer	
	Establishment of glycolysis pathway	
	Discovery of phosphorylase	Discovery of myosin ATPase
1940s		Purification of myosin & actin, Actomyosin+ATP→ superprecipitation,
		Dual action of ATP, Contraction of muscle fiber induced by Ca ion injection
1950s	Discovery of Phosphorylase kinase (Ph kinase)	Relaxation mechanisms
	<Ph kinase: the first protein kinase>	the essential relaxing factor
	Irreversible activation of Ph kinase with mM level Ca ion	
	Discovery of cyclic AMP <the first "second messenger">	
		Myofibril ATPase regulation by low concentrations of Ca ions
1960s		Ca-uptake by sarcoplasmic reticulum(SR): SR was identified as the relaxing factor
		Regulation of superprecipitation by low concentrations of Ca ions <the second "second messenger">
	In vivo study of phosphorylase activation upon muscle contraction	
1962	ATP consumption on contraction *in vivo*	
1964	Irreversible activation of Ph kinase with KAF + mM level Ca ions	
1965		Discovery of troponin
1967-8	Canal construction connecting these two currents	
	Reversible activation of Ph kinase by Ca ions at μM level (10^{-7}-10^{-5}M) [Q1 & 2]	
	<Ph kinase: the first protein kinase regulated by Ca ions>	
	Total activity of Ph kinase with the Ca ions fits well	
	to the *in vivo* conversion of phosphorylase *b* to phosphorylase *a* [Q3]	
	cyclic AMP potentiates the activation of Ph kinase with Ca ions [Q4]	
	<relationship between the second messengers>	
1968	Discovery of protein kinase A	
1973	Discovery of calmodulin	
1979	Discovery of protein kinase C	

Items colored blue and green belong to Currents 1 and 2 of muscular physiology, respectively. Items in the box are our discoveries. [Q1]–[Q4] correspond to [Question 1]–[Question 4] in Sections 6.2–6.5, respectively. Items colored in red belong to the signal transduction.

upon contraction. Thus, energy metabolism is much more important in the physiology of muscle than in that of other cell types. There must be a certain mechanism to restore ATP concentration that is closely connected with contraction. For the restoration, glycogen is the ultimate source of chemical energy. Thus, one of the very important questions in muscle physiology has been how glycogenolysis is regulated coupling with muscle contraction. I have to emphasize that in muscle, energy metabolism is not only the "housekeeping phenomenon", as in other cell types, but is also a cell function itself. In addition to the energy problem, various concepts on concurrent signal transduction including phosphorylation (for review[1]) and regulation with low concentrations of Ca ions (10^{-7}–10^{-5} M) (for review[2]) originated and developed from the studies of muscle physiology.

2. Beginning of study on energy metabolism and phosphorylation

2.1. Glycogenolysis and lactate formation on muscle contraction. Historically, the process involving glycogenolysis and lactate formation was initially considered as the mechanism of muscle contraction itself. Glycogen was discovered by Claud Bernard in the liver and muscle in 1853 and 1859, respectively. The decrease in glycogen content in the muscle fiber after muscle contraction was found by Nasse in 1869. The acidic substance was identified as lactic acid by Wislicenus in 1884, which had been known to be a characteristic product of milk fermentation (These were written according to Needham[3]).

In 1907, Fletcher and Hopkins[4] established the basis of the modern study of glycogen metabolism by quantitative analysis of the relationship between muscle contraction and lactate formation under anaerobic conditions. In 1914, Parnas and Wagner[5] showed that the decrease in glycogen content and production of lactate are stoichiometrically similar, suggesting the formation of lactate from glycogen. These phenomena were carefully studied by Meyerhof from 1920 to 1922 (for monograph[6]), who found that the amount of lactate produced is proportional to work and heat production upon muscle contraction and concluded that lactate production is essential for muscle contraction. This work was very highly appreciated and he was awarded with the Nobel Prize in 1922 together with A. V. Hill, who studied heat production upon muscle contraction (for monograph[7]). This shows how important the issue of glycogen metabolism was for classical muscle physiology.

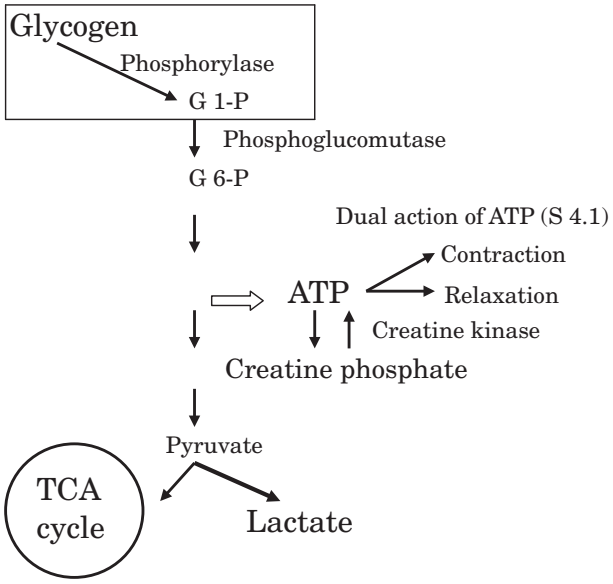

Fig. 1. Glycogenolysis and glycolysis pathways to lactate. The reaction in the box denotes glycogenolysis that is followed by glycolysis pathway in which G6-P is degraded to lactate. (S 4.1) means "see Section 4.1". TCA cycle: tricarboxylic acid cycle. ATP synthesized by degradation of pyruvate is not drawn, because the present review only deals with anaerobic contraction. For further details see the text.

Important phosphorylated low-molecular-weight substances related to glycogenolysis and glycolysis were discovered in muscle extracts in the following years, such as hexose mono- and diphosphates. Among them, creatine phosphate (phosphagen) was discovered in 1927 independently by Eggleton and Eggleton[8] and by Fiscke and Subarrow.[9] ATP was discovered in 1929 independently by Fiscke and Subarrow[10] and by Lohmann[11] of the Meyerhof school. Its molecular structure was determined by Makino in 1935.[12] However, the function of ATP in muscle contraction was not determined until a few decades later.

In 1930, a little-known Dane, Einar Lundsgaard, reported that the muscle of a frog poisoned with monoiodoacetic acid (MIAA) could contract for a while and then fell into final contracture, although lactate formation did not occur (for review[13]). The works of Meyerhof were rejected. However, despite this rejection, Meyerhof enormously contributed to the establishment of glycolytic pathway (Fig. 1). In 1932–1934, Lundsgaard reported that in MIAA-intoxicated muscle, creatine phosphate was reduced and finally disappeared following repeated contractions, resulting in the contracture of the muscle. Notably, he observed that ATP concentration also

markedly decreased in MIAA-intoxicated muscle immediately before the muscle fell into final contracture; however, he did not pay much attention to his own discovery and described this very briefly.[13] In any case, this paper was written in Danish, and researchers worldwide did not notice it until it was translated into English in 1969.[13] It was later found that MIAA is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase in the glycolysis pathway.

In 1934, creatine kinase was discovered by Lohmann.[14]

$$\text{creatine phosphate} + \text{ADP} \underset{\longleftarrow}{\overset{\text{creatine kinase}}{\longrightarrow}} \text{creatine} + \text{ATP}$$

On the basis of this reaction, many researchers assumed that ATP consumed upon muscle contraction is immediately restored from creatine phosphate by moving the phosphate group and that the consumption of ATP could not be observed.

On the basis of Lohmann's equation,[14] Parnas[15] proposed the concept of "transfer of phosphate residue". He recognized the special significance of the transfer of the phosphate group. Indeed, the concept of biochemical phosphorylation originated from the study of sugar metabolism. It was expanded to a new concept by Lipmann[16] in 1941, that is, ATP has specific phosphate bonds and acts as a universal energy donor by releasing a γ-phosphate residue. Later, the phosphate residue transfer concept was further expanded and changed to a new concept that phosphate residue transferred from ATP to a protein changes the function of the protein.[1] Phosphorylation of proteins became one of the most important concepts of signal transduction and thus, muscle energetics was the cradle of a large branch of current cell biology.

Another question was which one of creatine phosphate and ATP is the direct energy source for contraction. From the 1930s, it was assumed that energy contained in either ATP or creatine phosphate must be used for muscle contraction, because it was known that a large amount of energy is released upon hydrolysis of these substances. On the basis of some additional circumstantial evidence described in Section 4.1, biochemists considered in the 1940s that ATP was the direct energy source of contraction. However, direct evidence of an *in vivo* decrease in ATP concentration upon contraction was not obtained for decades. Thus, researchers were unable to theoretically exclude the possibility that creatine phosphate was the direct energy source of contraction.

2.2. Use of ATP for muscle contraction. By the end of the 1930s, the pathway from glycogen to lactate *via* glycolytic cascade was mostly elucidated; ATP is generated *via* this pathway during anaerobic contraction (Fig. 1). There still remained two important questions. Question [I]: is the direct energy source of muscle contraction indeed ATP? Question [II]: if this is the case, how is glycogenolysis regulated in connection with muscle contraction to supply ATP constantly? The latter question is the fundamental topic of the present review.

For Question [I], it is known that ATP is the universal energy donor and that, as described below, ATP is always required for *in vitro* contraction and creatine phosphate cannot replace ATP in this reaction. However, there was no evidence that the concentration of ATP in muscle changes upon muscle contraction. Therefore, many physiologists were skeptical about the consumption of ATP *in vivo*. Indeed, even in 1956, Heilbrunn,[17] a leading American biologist, wrote, "Perhaps it is this (ATP) energy that is responsible for the contraction of muscle; such indeed is the opinion commonly held at the present time, although there is little or no direct evidence to prove it."

Finally, in 1962, Cain *et al.*[18] of R. E. Davies' group treated frog muscles with dinitrophenol, a potent uncoupling agent of oxidative phosphorylation, which exerted no effect on muscle contraction and creatine phosphate consumption. These muscles were further treated with 1-fluoro-2,4-dinitrobenzene, which was known as a specific inhibitor of creatine kinase. They stimulated the double-intoxicated muscles to contract. After repeated contraction by electrical stimulation, when the muscle became nonresponsive and fell into contracture, it still contained the normal concentration of creatine phosphate. Instead, inorganic phosphate concentration increased and ATP concentration decreased. The amount of ATP consumed was equivalent to that expected from the amount of creatine phosphate that decreased upon the contraction in nonintoxicated muscle. The hypothesis of ATP consumption upon muscle contraction was proved. The previous observations of Lundsgaard were correct.[13]

There is an additional question that glucose from circulating blood may be absorbed into the muscles during contraction and may contribute to the production of ATP. This is indeed the case, but its effect is much smaller than that of glycogenolysis and is neglected in the following discussion.

This section is concluded with Question [I]: ATP ($\sim$3 mM[19]) is consumed during muscle contraction but its concentration is maintained by simultaneous transfer of "high-energy-bond" phosphate from creatine phosphate. However, creatine phosphate is also limited in amount ($\sim$20 mM[19]) and must be supplied by glycogenolysis under anaerobic conditions. As we have seen, in normal muscle, ATP concentration remains unchanged during muscle contraction, preventing muscle contracture owing to ATP depletion. There must be a stable mechanism that keeps the concentration of ATP constant. We now confront Question [II]: how is glycogenolysis regulated during muscle contraction that supports the maintenance of ATP concentration. This is the main topic of this review.

3. Phosphorylase and cyclic AMP, the first "second messenger"

3.1. Phosphorylase degrades glycogen. In 1929, C.F. Cori and G.T. Cori[20] showed that lactic acid formed upon muscle contraction rapidly diffuses out of the muscle into the blood stream, which carries it to the liver, where lactic acid is converted to glucose then to glycogen. Glycogen in the muscle arises from glucose produced from the breakdown of liver glycogen and is carried to the muscle by blood. The cyclic process summarized below is termed the Cori cycle.

$$\begin{array}{ccc} \text{Muscle glycogen} & \longrightarrow & \text{Blood lactic acid} \\ \uparrow & & \downarrow \\ \text{Blood glucose} & \longleftarrow & \text{Liver glycogen} \end{array}$$

Epinephrine was found to accelerate the Cori cycle in the direction of muscle glycogen to liver glycogen and to inhibit it in the direction of blood glucose to muscle glycogen; as a result, sugar accumulates in the blood.

In 1935–1937, phosphorylase was discovered by Parnas and coworkers[21),22)] and Cori *et al.*[23),24)] Its catalytic reaction is as follows.

$$\text{Glycogen} + \text{Pi} \xrightarrow{\text{Phosphorylase}} \text{Glycogen}^* + \text{G1-P}$$

(Pi, inorganic phosphate; Glycogen*, glycogen minus one glucose residue; G1-P, glucose 1-phosphate)

The structure of glycogen and its breakdown are explained as follows: Glycogen is characterized by a highly ramified structure, in which straight chain arrays of 11 to 18 D-glucopyranose units (in α(1 to 4)-glycosidic linkage) are cross-linked by α(1 to 6)-glucosidic bonds. Phosphorylase "phosphorolyses" its 1,4 bond to form G1-P. In addition, debranching enzyme cuts the terminal 1,6 bonding of glycogen. Thus, glycogenolysis by phosphorylase can continue.

In 1938, Cori *et al.*[25)] showed that G1-P is converted to G6-P by phosphoglucomutase and the glycogenolysis product G1-P can enter into the glycolysis pathway (Fig. 1). At this time, the glycolysis pathway was almost established and G6-P was known to be the source of ATP (Fig. 1). Although the metabolism map from glycogenolysis to lactate production was almost completed, Question [II], that is, how this process is regulated upon muscle contraction, remained to be solved.

In 1942, when phosphorylase was crystallized by Green *et al.*,[26)] it became clear there are two types of phosphorylase, phosphorylase *b* (Ph *b*), which absolutely requires AMP for its activity, and phosphorylase *a* (Ph *a*), which shows activity in the absence of AMP. The molecular weights of Ph *a* and Ph *b* are 495 and 242 kDa, respectively. Subsequently, phosphorylase rupturing (PR) enzyme that convert Ph *a* to Ph *b* was discovered by Keller and Cori.[27)] After many studies, the possibility was finally excluded that AMP is a prosthetic residue that serves as a regulatory factor of phosphorylase.

3.2. Cyclic AMP. The decrease in glycogen content following epinephrine treatment was observed by Lasser in the muscle in 1920[28)] and by Cori and Cori in the liver in 1928.[29)] In 1951, Sutherland and Cori[30)] showed that this effect of epinephrine and also glucagon on the liver was due to the activation of phosphorylase.

In 1957–1958, Sutherland's group[31)–33)] made the following discoveries. When they studied the effect of epinephrine on the activation of liver phosphorylase using cell-free homogenate, they found that epinephrine was not effective on the supernatant containing phosphorylase. Next, they found that epinephrine reacted with the granular fraction, resulting in the production of an unknown heat-stable substance that in turn activated phosphorylase. Soon, this activating substance was identified as adenosine 3′,5′-monophosphate (cyclic AMP). Independently, Cook *et al.*[34)] found that cyclic AMP was also chemically produced from ATP in the presence of $Ba(OH)_2$. These groups[34),35)] exchanged their respective samples for biological and chemical assays, and agreed that the samples were identical.

Later, it was found that cyclic AMP was produced from ATP as catalyzed by adenyl cyclase present on the cell membrane that was stimulated by epinephrine or glucagon (for review[36)]). Adenyl

cyclase[37] was also stimulated by norepinephrine or isoproterenol. This stimulation was inhibited by a β-blocker, dichloroisopropylalterenol. The ring structure of cyclic AMP was opened by phosphodiesterase to form AMP, and the stimulatory effect was lost. Phosphodiesterase activity was inhibited by xanthine derivatives, such as theophylline.

Thus, the following pathway was established. A bioactive extracellular substance acts on the enzyme present on the cell membrane to produce an intracellular substance different from the original extracellular substance. The intracellular substance conducts the information to its target for activation. Cyclic AMP is the first "second messenger" discovered (for monograph[38]). Later, the term second messenger was expanded to include substances that are not necessarily related to the cell membrane enzyme, as long as they convey a signal transduced from an external first message.

4. Introducing the concept of regulation by low concentrations of Ca ions to the field of muscle physiology

Before dealing with further developments in studies of glycogenolysis, I would like to discuss another current of muscle physiology, namely, the mechanism of muscle contraction. In particular, I will describe how the effect of low concentrations of Ca ions on muscle contraction was discovered.[2]

Today, the intracellular concentration of free Ca ions in the static state of not only muscle fibers but also most cell types is widely believed to be at approximately micromolar (μM) level or lower. However, before the early 1960s, until the regulatory mechanisms of muscle contraction were established, the role of Ca ions at such low concentrations was not well understood. The reasons for this were that chelating compounds for preparing metal ion buffers (for monograph[39]) were not available and that Ca ions contaminating the reagents and samples were not considered at that time. Many researchers, particularly biologists and biochemists, considered that bioreactions should be conducted using Ca ion concentrations at the millimolar (mM) level. This difference in concentration of Ca ions is the essential point of discussion in this review.

4.1. Beginning of the study of the mechanisms of muscle contraction. For a long time, the chemistry of muscle contraction was studied as a subject of energy metabolism. Back in 1939, Engelhardt and Lubimova[40] reported that myosin has ATPase activity. Myosin preparations at that time contained various proteins including actin and other regulatory proteins that were later discovered.

In 1940–1943, in the midst of World War II, Albert Szent-Györgyi and his Hungarian group founded the basis for the modern biochemical or *in vitro* study of muscle contraction. These brilliant contributions were later summarized in his monograph published in 1947 (1st ed.) and 1951 (2nd ed.).[41] Both editions of this book tremendously influenced the development of muscle research in Japan.

The contributions of Szent-Györgyi group were as follows:[41] (1) Straub of this group separated and purified actin and myosin. However, their actin preparations must have contained other minor regulatory proteins that would be discovered later. The mixture of actin and myosin was termed actomyosin. (2) Szent-Györgyi found that when ATP-Mg was added to actomyosin, "superprecipitation" occurred. This is the precipitation of proteins specific to actomyosin that contained an extraordinarily small amount of water in it. He intuitively considered this to be "muscle contraction *in vitro*." This interpretation was later supported by an electron microscopy study (for review[42]). The discovery of superprecipitation was one of the foundations of modern muscle contraction study. (3) However, somewhat bewildering phenomena were observed, namely, in the presence of higher concentrations of ATP, actomyosin remains to be separated (relaxation), whereas in the presence of lower concentrations of ATP, it superprecipitates (contraction). This is called the dual action of ATP. The actual concentrations of ATP for these reactions differ depending on the ionic strength of the buffer solution. If superprecipitation is equivalent to contraction, the process should be reversible because contracted muscle must relax. However, Szent-Györgyi did not succeed to show its reversibility. This is historically noteworthy, because the studies on the question how muscle relax lead to the elucidation of the regulatory mechanism of muscle contraction (see Section 4.4 and below). (4) Szent-Györgyi developed "glycerol-extracted psoas" or "glycerinated fibers". These are prepared from long muscles like illiopsoas (This is only described in the 2nd edition of Ref.[41]). Both ends of the muscle fibers are tied with threads to a glass rod and stored in 50% glycerin placed in a freezer at the temperatures such as $-20\,^{\circ}\mathrm{C}$, for a few days or several months depending on the experimental needs, to lyse the cell membrane and release various soluble proteins and

small molecules. After the storage period, the fibers still had contractility in a buffer solution containing ATP and other reagents. When the fibers contract, the shortening of the fibers and the force generated can be recorded using experimental physiological devices. This was used as an *in vitro* model of contractile elements that may still retain their organized structures that living muscles possess.

Later, Natori[43] developed a method of preparing a skinned fiber. He immersed a single toad muscle fiber in whale oil, and its cell membrane was mechanically removed with a knife. This skinned fiber is much fresher than glycerinated fibers and is considered to be an experimental model close to a living muscle fiber. This preparation method was improved in the later half of the 1960s by immersing the muscle fiber in an ethylene glycol-bis(β-aminoethylether)-N,N′ tetraacetic acid (EGTA) buffer in place of oil (for review[2),44]).

4.2. Forerunners of Ca ion-theory of muscle contraction. In 1940, Heilbrunn[45] found that when isolated frog muscle fibers are immersed in $CaCl_2$ solutions, they shorten rapidly and extensively. Then, Kamada and Kinoshita[46] and Heilbrunn and Wiercinski[47] independently showed in 1943 and 1947, respectively, that localized contraction was induced in a fresh single muscle fiber that then slowly longitudinally spread, when its sarcolemma was injured by pinching with forceps or tearing with injection pipette. This phenomenon was observed solely in a solution that contained Ca ions. The latter[47] wrote that among the inorganic positive ions, only Ca ions induced contraction. They further wrote as follows: "Many types of stimulation cause a muscle fiber to contract. We believe that essentially all these diverse types of stimulation cause a release of calcium ion from the surface or outer region of the cell and that this calcium then enters the cell and produce the response. If the presence of free calcium ion is primarily responsible for contraction of muscle protoplasm, then it is logical to assume that, in general, various types of chemical stimulation cause a release of calcium ion into the interior of the muscle cells. Thus, if a muscle is immersed in a potassium chloride solution, the contracture produced by such a solution may well be due to a release of calcium." These statements are very interesting. However, we have to note that they were not described on the basis of experimental evidence but only on assumption.

Later, in 1956, Heilbrunn[17] explained his Ca hypothesis of muscle contraction in his monograph. He wrote that Ca ions were the trigger of muscle contraction, and that their effective concentration was considered to be lower than 0.2 mM (the evidence for this was not shown). In any case, his contraction model appears to be similar to a model of blood clotting. Although he considered that relaxation is important, he was unable to explain how the effect of Ca ions on contraction is cancelled for relaxation. On the other hand, although he theoretically well understood Szent-Györgyi's works, he did not appreciate them. He wrote, "Obviously, the living muscle does not behave like glycerinated fibers do, and it is the living muscle rather than the dead muscle that we are primarily concerned with." Even in the middle of the 1950s, the understanding of muscle contraction was markedly different between Heilbrunn, a biologist, and Szent-Györgyi, a biochemist, who were renowned scientists of the same generation.

4.3. How the electrical signal is conducted from the cell membrane to the contractile elements. The signal for contraction is conducted by acetylcholine released from nerve terminals that diffuses across the synapse cleft to the end plate on the muscle side (Fig. 2). Acetylcholine induces electrical depolarization on the endplate, which induces the excitation of the adjacent cell membrane. Electrical excitation spreads throughout the cell membrane. The problem in the late 1940s and 1950s was how the signal of electrical excitation is conveyed to contractile elements, namely, myofibrils mainly composed of actomyosin.

One of the simple models is that upon the electrical excitation of the cell membrane, a certain signal substance enters from the extracellular space into the cytoplasm through the cell membrane or is released from the cell membrane and diffuses to reach the contractile elements as described by Heilbrunn and Wiercinski.[47] In 1948, Hill[48] pointed out "if the twitch of a muscle fiber is assumed to involve the contraction of the whole of its contents (author's note: myofibrils), diffusion from the outer surface could not be fast enough to account for observed speeds of contraction." This was not compatible with the claim of Heilbrunn.

In 1955, Andrew F. Huxley and Taylor[49] showed that when a microelectrode was placed on a tiny spot on a frog muscle cell membrane facing a Z-band of myofibrils and the spot was electrically stimulated, local contraction occurred that was limited to the two sarcomeres adjacent to the Z-band that was stimulated. They assumed from this observation that there must be conducting systems of

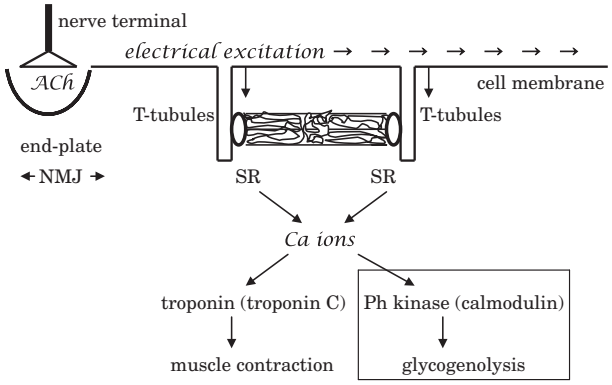

Fig. 2. Mechanisms of muscle contraction and glycogenolysis. Evidence shown in the box is described in Section 6.2. From the nerve terminal, the first signal or "the first messenger" of acetylcholine (ACh) is transmitted to the end plate of the neuromuscular junction (NMJ). This signal is transduced into electrical excitation that is conducted throughout the cell surface membrane, including the T-tubules. This signal is conducted to SR, where it is again transduced into "the second messenger" Ca ions. Ca ions are released into the cytoplasm and diffuse to troponin and Ph kinase for activation. Thus, the contractile system and glycogenolysis system are simultaneously activated. When Ca ions are taken up by SR and their cytoplasmic concentrations are decreased, Ca ions are detached from troponin and Ph kinase and these systems are inactivated.

the electrical stimulus deep into the muscle fiber along the Z-band.

Morphologists took the challenge to elucidate the structural basis of this effect (for review[50]). They found the T-tubules, which are long pipelike cul-de-sac structures penetrating deep into the muscle fiber surrounding myofibrils at the Z-band level and which have their opening localized at the cell membrane facing Z-band in the frog muscle (Fig. 2). T-tubules extensively branch out and attach to the sarcoplasmic reticulum (SR) that longitudinally entangles along the sarcomere. It is noteworthy that the opening of T-tubules is located at the cell membrane facing the Z-band in the frog muscle. Thus, taken together with other observations, the local stimulatory effect is conducted deeply inside the muscle fiber (for review[51]). Thus, the connection between T-tubules and SR became clear, although at that time the physiological roles of SR were not yet understood.

4.4. SR is the intracellular depository of Ca ions. Scientists succeeding Szent-Györgyi first confronted a difficult question that he did not answer. It concerned the mechanism of relaxation. Muscle contraction should be reversible, namely once contracted muscle should relax. In 1951, Bozler[52] found that the glycerinated muscles once contracted by the addition of ATP were not relaxed by the removal of the remaining ATP, but were relaxed by the further addition of ATP. This was a rediscovery of the dual action of ATP[41] (see Section 4.1). Does the change in ATP concentration serve as the regulator of the contraction–relaxation cycle? As stated in Section 2.2, the concentration of ATP does not change *in vivo* during the cycle. Thus, ATP is not the regulating substance of contraction.

In 1951, Marsh[53] showed that a particular relaxing factor is present in the muscle extract. The identification of this factor was pursued by several research groups. Among them, Ebashi, who started his work before 1952, reported in 1955[54] and in succeeding years that the essential relaxing factor is the Kielley-Myerhof ATPase particle, whose function is promoted by a fraction containing an ATP-reproducing system (for review[55]).

Bozler[56] showed in 1954 that ethylene diamine tetraacetic acid (EDTA) in the presence of ATP and Mg relaxed the contracted glycerinated muscle. Watanabe[57] in 1955 reported that the effect of EDTA on the contracted glycerinated muscle resembled that of the relaxing factor. However, Bozler considered that EDTA cancelled the function of Ca ions that was present firmly bound to contractile proteins, and Watanabe claimed that EDTA acted on Mg that was also firmly fixed to the proteins. Reading these reports, Ebashi considered that their interpretations were wrong, because, in principle, chelating compounds provide all the ligands to divalent cations and can bind only to free ions. Thus, chelating compounds cannot work on Ca or Mg ions bound to the proteins. He confirmed his idea using Dowex-1, which does not bind to myofibrils. On this basis, he considered that the relaxation was due to the effect of chelating agent to take out divalent cations from the contractile elements (for review[58]).

In 1960, Ebashi[59] examined the effects of various types of chelating compounds on relaxation in the presence of 10 mM Mg ions and found that there was a linear relationship between the Ca ion binding and the relaxing effects on glycerinated fibers. He considered that these results suggest that the regulating substance for contraction is not Mg ions but Ca ions.

During his stay in Lipmann's laboratory in New York in 1958–1959, Ebashi obtained a pure preparation of the vesicular relaxation factor and found that it binds to Ca ions in the presence of Mg-ATP, suggesting that the relaxing factor takes up Ca ions by consuming ATP.[60] Moreover, on the basis

of Palade's electron microscopy observations of Ebashi's preparation of the vesicular relaxing factor, Ebashi and Lipmann[60] concluded that the factor is composed of SR. Thus, SR was determined to be the depository of Ca ions. It became clear that relaxation is induced by the deprivation of Ca ions from myofibrils, by decreasing the Ca ion concentration in the cytoplasm *via* the uptake of Ca ions by SR.

These works showed that SR is the depository of Ca ions and takes up Ca ions from the cytoplasm. However, the release of Ca ions from SR was not directly proved. Nevertheless, on the basis of Ebashi's findings, most researchers at that time concurred with the assumption that SR can also release Ca ions on the basis of other indirect evidence. This assumption was later corroborated by the following observations: Weber and Herz[61] showed in 1986 that caffeine induces Ca ion release from SR. Caffeine had been known to induce muscle contraction without excitation of the cell membrane. Somlyo *et al.*[62] found in 1981 that upon tetanus induction, Ca ion concentration decreases in SR and increases at myofibrils. They interpreted these findings to mean that Ca ions are released from SR and diffuse to the myofibril region.

4.5. Contraction is induced by low concentrations of Ca ions. The issue on whether contraction of myofibrils or actomyosin can be induced by low concentrations of Ca ions was studied in parallel with the above studies. It might be unbelievable for present-day researchers that until the early 1960s, the works of Kamada and Kinoshita[46] and Heilbrunn and Wiercinski[47] were not known among the researchers of the chemistry of muscle contraction.

Annemarie Weber[63] reported in 1959 that myofibril and actomyosin ATPases are activated by low concentrations of Ca ions, respectively. From the viewpoint of classical enzymology, these ATPases are not pure enzymes nor catalysts in the classical meaning, such as Ph kinase, but they are complexes composed of many proteins and the formation of ADP and Pi is due to the cleavage of ATP resulting from the use of energy for contraction. Therefore, these ATPases are the index of muscle contraction, and her reports were the first to show that low concentrations of Ca ions induce muscle contraction.

On the other hand, in 1961, Ebashi[64] developed a method to spectrophotometrically measure the superprecipitation of actomyosin suspended in a buffer, and this development promoted his studies. He removed Ca ions contaminating all the samples and reagents and examined the effect of low concentrations of Ca ions on superprecipitation. He found that superprecipitation is regulated by Ca ions at concentrations ranging from 2×10^{-7} to 5×10^{-6} M. This range roughly coincided with the range in which ATPases were regulated as shown by Weber.[63] In 1968, Ebashi and Endo[2] showed that a frog skinned fiber in which the cell membrane was mechanically removed in EGTA buffer responded to low concentrations of Ca ions that were similar to the concentrations that induced superprecipitation, although the concentrations needed for the frog skinned fiber to respond were slightly higher than those required for the superprecipitation of rabbit actomyosin.[64] Therefore, the concentration of Ca ions required for muscle contraction was very low. This suggests that the concentration of Ca ions in the cytoplasm during relaxation is extremely low and increases up to approximately 3×10^{-5} M, as determined from their data. Indeed, Konishi and Baylor[65] later reported that the peak concentration of Ca ions during contraction in living frog muscles is 1.3×10^{-5} M.

4.6. Troponin: Ca ion receptor on thin filaments. With the improvement of the method of preparing actin, the regulation of the superprecipitation of actomyosin by Ca ions became uncertain. Actomyosin composed of purer actin and myosin was not amenable to Ca ion-regulation. On this basis, Ebashi found that a new substance other than actin and myosin was necessary to restore Ca ion-regulation. At first it was considered to be a protein resembling tropomyosin in amino acid composition and was named native tropomyosin. In 1965, Ebashi and Kodama[66] finally discovered a new protein, troponin, that restores Ca ion-regulation when added to actomyosin together with tropomyosin. It turned out that native tropomyosin is a complex of tropomyosin and troponin. By 1967, at least the following had become clear: troponin binds to Ca ions[67] and together with tropomyosin, troponin decorates actin filaments.[68] Troponin inhibits the interaction between actin and myosin in the absence of Ca ions, but upon the binding of troponin to Ca ions, this inhibition is disrupted and contraction occurs.[2] This is the molecular basis of Ca ion-regulation for muscle contraction.

In 1968, Ebashi's study was forced to be interrupted by the student activism in universities occurring nationwide that lasted for a few years. His laboratory was blockaded. During this period, it turned out that troponin is composed of multiple components. Hartshorne and Mueller[69] in 1968

described troponin C that binds Ca ions and Schaub and Perry[70] in 1969 found troponin I that inhibits the interaction of actin to myosin. Finally, in 1971, immediately after the troubles in universities were over, Ebashi *et al.*[71] reported troponin T that binds tropomyosin. It is now believed that troponin is composed of these three subunits.

In conclusion, the substance that regulates muscle contraction is low concentration Ca ions. Electrical stimulation of the cell membrane is conducted *via* T-tubules to SR. Ca ions are released from SR and their concentration in the cytoplasm changes from 10^{-7} to 10^{-5} M, for example. Ca ions diffuse to the thin filaments and bind to troponin C, resulting in contraction (Fig. 2). When Ca ions are taken up by SR and the concentration of Ca ions in the cytoplasm decreased, Ca ions uncouple from troponin C, resulting in relaxation. In other words, increasing and decreasing the Ca ion concentration in the cytoplasm causes contraction and relaxation, respectively, and after the relaxation, there remains no after-effect on contractile systems in terms of Ca ion-regulation. Here, we must emphasize that the effect of Ca ions is reversible in terms of contraction.

5. Phosphorylase kinase and glycogenolysis

Let us again consider Question [II] (see Section 2.2): how the glycogenolysis is regulated upon muscle contraction.

5.1. Discovery of phosphorylase kinase. Back in 1955, in the year that Ebashi reported the essential relaxing factor,[54] Edwin G. Krebs and Edmond H. Fischer[72),73] discovered a new enzyme in muscle extract. They found that the content of Ph *b* was high in the extract solution that was obtained with buffer solution containing EDTA, whereas it was low in the extract solution obtained with buffer solution without containing EDTA. They assumed that in the reaction that converts Ph *b* to Ph *a*, a divalent cation may be involved.

On the basis of this assumption, they obtained the following results: 1) When any one of the divalent cations Ca, Sr, Ba and Mn was added to a fresh muscle extract at a concentration of 1 mM (To readers, please pay attention to the concentration of the cation throughout this review.) and the extract was incubated, the percentage of Ph *a* activity increased. "Percentage of Ph *a* activity" means the activity of Ph *a* (measured in the absence of AMP) × 100/total phosphorylase activity measured in the presence of AMP. 2) From a muscle extract that was stored at 3 °C for 24 hours, or dialyzed, or treated with active carbon, the effect of a divalent cation was lost. However, when any one of these extracts was incubated with ATP and Mn ions, Ph *a* content increased. 3) The phosphorylase-activating effect was not observed when the fraction precipitated at pH 5.8 was removed from the extract. However, this effect was restored by returning the precipitate to the precipitate-free fraction. They examined some natures of the precipitate and concluded that it is an enzyme that converts Ph *b* to Ph *a* in the presence of ATP and 1 mM divalent cation. This enzyme was termed "converting enzyme".

In 1956, one year before the discovery of cyclic AMP, Rall *et al.* of Sutherland's group[74] using liver and Krebs and Fischer[75] using muscle independently showed that the converting enzyme transferred γP from ATP to Ph *b*, which was converted to Ph *a*. As a reverse reaction (see Section 3.1), Ph *a* was reconverted by PR enzyme to Ph *b* that removed Pi (Pi: inorganic phosphate) from Ph *a*. Thus, the converting enzyme and the PR enzyme were renamed phosphorylase *b* kinase (later again renamed phosphorylase kinase: Ph kinase) and phosphoprylase phosphatase (Ph phosphatase), respectively.

Furthermore, because the concentration of phosphate residues was 4 moles/1 mole protein, the following equation was proposed taking the molecular weights of Ph *a* and *b* into consideration.[26]

$$2\text{Ph } b + 4\text{ATP} \xrightarrow{\text{Ph kinase}} \text{Ph } a + 4\text{ADP},$$

and the reverse equation is

$$\text{Ph } a \xrightarrow{\text{Ph phosphatase}} 2\text{Ph } b + 4\text{Pi}.$$

This Ph kinase reaction later turned out to be the first case of the reaction of a protein kinase that phosphorylates a protein resulting in the change in the function of the protein. However, protein phosphorylation itself had already been reported by Burnet and Kennedy[76] in 1954, that is, the phosphorylation of casein by casein kinase. However, changes in the function of casein were not known.

5.2. Studies of phosphorylase activation in muscle contraction *in vivo*. In 1956, Cori[77] reported that the content of Ph *a* increased when muscle contracted, although his data largely fluctuated. However, in 1962, Danforth *et al.*[78] clearly showed that the percentage of Ph *a* activity in the relaxed frog muscle was 2.8%, whereas it linearly increased to 80% after about 2.5 sec of continuous electrical stimulation of the muscle, as determined from their data. During relaxation after a sustained contraction, the content of Ph *a* decreased in about

60 sec with a time course tracing a hyperbolic-like concave curve. The question was whether the conversion of Ph *b* to Ph *a* upon muscle contraction was due to the activation of Ph kinase or to the inhibition of Ph phosphatase. They analyzed their results and concluded that the activity of Ph kinase increased, whereas that of Ph phosphatase did not change during contraction. The next question was what activates Ph kinase upon muscle contraction.

5.3. Krebs' studies on the activation of Ph kinase by Ca ions. In 1958, Fischer and Krebs[79] developed a new method of crystallization of Ph *b* with high yield. This promoted the following studies.

In 1959, Krebs *et al.*[80] concentrated on the use of Ca ions among the divalent cations for the activation of Ph kinase on the basis of Heilbrunn's Ca hypothesis of muscle contraction and reported the following: 1) When partially purified Ph kinase was incubated with 3×10^{-3} M Ca ions, the enzyme remained inactive at pH 7.0, whereas it was highly active at pH 8.3. 2) The activity reached a maximum when the enzyme was incubated at pH 9.0 for 3–5 min. 3) Once Ph kinase was activated, it was not inactivated by the addition of EDTA with twice the amount of Ca ions, or even when it was dialyzed in EDTA solution. 4) In contrast, the activity of once-activated Ph kinase was lost when it was incubated for a long time together with Ca ions at this concentration.

The aim of Krebs must have been to clarify the mechanism of Ph kinase activation upon muscle contraction. While the above studies were in progress, Krebs knew the works of Heilbrunn.[17] However, immediately after the publication of these studies, Krebs must have been aware of the works of Ebashi[59] and Weber[63] on the effect of low concentrations of Ca ions on muscle contraction, judging from the references cited in his succeeding papers. In spite of the knowledge, he did not pay attention to the difference in Ca ion concentration. He did not use a μM-level concentration of Ca ions in his actual studies, probably because biochemical researchers at that time customarily used inorganic ions at the mM-level without taking note of biological evidence.

In August 1964, Meyer *et al.* of Krebs' group[81] separated a Ph kinase fraction into two: one contained Ph kinase and the other contained a protein factor that activated Ph kinase in the presence of mM-level Ca ions at alkaline pH. They named this protein factor "kinase activating factor (KAF)". Other divalent cations listed above were effective when used in place of Ca ions. On the other hand, when Ph kinase was treated with trypsin, partially digested Ph kinase became active. Therefore, they suspected, but did not conclude, that KAF was a Ca-stimulated proteolytic enzyme (see Section 7.1.4).

5.4. Further studies of phosphorylase activation in muscle contraction *in vivo*. In October 1964, Danforth and Helmreich[82] reported the relationship between the K ion concentration in Ringer solution (depolarization of cell membrane) and the percentage of Ph *a* activity. When a frog sartorius muscle was incubated for 10 min in Ringer solution having various concentrations of K ions that substitute Na ions, the percent activity Ph *a* increased with the concentration of K ions. (This experiment was performed under the conditions that were not necessarily well documented.) On the other hand, in 1960, Hodgkin and Horowicz[83] showed the relationship between K ion concentration in Ringer solution and tension generated in a single fiber. Although Danforth and Helmreich did not refer to this paper, the curves of these reports were strikingly similar. The distinct range of concentrations of K ions that activated the contraction similarly promoted the conversion of Ph *b* to Ph *a*. Naturally, they referred to the works of Krebs' group. However, they correctly understood that Krebs' observations could not represent physiological conditions because of the irreversibility of activation by Ca ions and stated, "More evidence is necessary before Ca ions can be assigned a role in the activation of phosphorylase kinase *in vivo*." However, they also did not pay attention to the concentration of Ca ions.

Danforth and Helmreich further showed that the percentage of Ph *a* activity increased in the muscle treated with caffeine. In 1968, one year after publication of our first work described in the next section, caffeine was found to induce the release of Ca ions from SR.[61] Taking all above stated evidence together, the glycogenolysis regulation mechanism should work after Ca ions are released for contraction. Retrospectively considered, in 1965, when I started my study on Ph kinase, circumstantial evidence for the regulatory mechanism of glycogenolysis on muscle contraction had fairly well been obtained. However, the final biochemical evidence was lacking.

6. Reversible Ph kinase activation by low concentrations of Ca ions

For my mentor, Professor Ebashi, who for a long time considered the role of low concentrations of Ca ions in muscle contraction and also the reversibility

of contraction, it was immediately clear that the biochemical works of Krebs' group[81] on phosphorylase kinase with KAF and high concentrations of Ca ions ($\sim 10^{-3}$ M) did not represent physiological reactions, because in their works (1) the concentration of Ca ions required for Ph kinase activation was too high and (2) the activation process was irreversible, as also pointed out by others.[82]

At the end of 1965, when I consulted with Professor Ebashi about my doctoral thesis, he happened to mention Krebs' work while he was explaining other projects. Immediately, I asked him whether I could study this topic. He was rather surprised, and told me "I have neither specialized knowledge nor experimental experience on glycogenolysis. If you wish to study this topic, you will have to search for the literature related to this topic and build up all the necessary bases of this study by yourself. Will you still do it?" "Yes, I will." I answered without any hesitation. He accepted my proposal and only pointed out Krebs' shortcomings, (1) and (2) described above. Naturally, at that time, I had no experience of scientific research.

Soon, I started studying papers and working on the crystallization of Ph *b* from the rabbit muscle, as a substrate of Ph kinase. Professor Ebashi gave me the complete freedom to try as I wished. I wandered alone in the academic wilderness by trial and error. Sometimes, I orally and briefly reported to Professor Ebashi how my experiments were doing, but he hardly discussed this problem with me. He helped me only when I asked for suggestions of my mentor on experimental procedures. However, I did not feel at all that I was spiritually alone. He was always warm and kind. Moreover, it was clear to me that one of the most important suggestions in research is to give strict initial questions, and that he wished me to complete all of these works by myself. Although there was almost no background on glycogen metabolism in Professor Ebashi's laboratory, there was much top scientific knowledge on muscle contraction that may not have been available in any other contemporary laboratories. This was a great advantage for me to carry on with my work. I spent solid and satisfactory days engrossed in research. After nearly ten months, I obtained the initial results.

6.1. Analysis of Krebs' model of Ph kinase assay. Before describing our works, it is necessary to analyze Krebs' model of Ph kinase assay, which was divided into three steps,[84]

[Step 1] Ph kinase activation reaction $-\langle$dilution and transfer$\rangle\rightarrow$ [Step 2] Ph kinase assay, converting Ph *b* to Ph *a* $-\langle$dilution and transfer$\rangle\rightarrow$ [Step 3] Phosphorylase assay.

Here, $\langle$dilution$\rangle$ means to stop the enzyme reaction of the previous step by diluting an aliquot of the reaction mixture with a given buffer, and $\langle$transfer$\rangle$ means to transfer an aliquot of the diluted sample to the assay medium of the next step as the enzyme. Concretely, [Step 1] is the main reaction in which Ph kinase is incubated with Ca ions and KAF. After a certain time of incubation, an aliquot is diluted. In [Step 2], an aliquot of the diluted sample is incubated with Ph *b* for conversion of Ph *b* to Ph *a*. In [Step 3], the activity of Ph *a* converted by the action of Ph kinase in [Step 2] is assayed.

I interpreted Krebs' model as follows. Ca ions must react at [Step 1], and Ph kinase activated by KAF and Ca ions is transferred to [Step 2] in which the activation of Ph *b* occurs. Unless Ca ions irreversibly affect the Ph kinase molecules in [Step 1], one cannot detect the incubation effect on Ph kinase in the following steps. This is a very important point of his strategy. We have to note that in his model, Ph kinase and Ph *b* are incubated together only in [Step 2] without controlling the concentration of Ca ions. Because KAF, which was suspected to be a Ca ion-stimulated proteinase, partially digests and activates Ph kinase in the presence of a high concentration of Ca ions in [Step 1], the activation effect on Ph kinase can be transferred to [Step 2]. In contrast, if reversible activation occurs in [Step 1], the activation effect would not be carried over to [Step 2], because the Ca ion concentration changes in the reaction mixture of [Step 2]. Thus, it was clear that Krebs did not pay attention to the effects of Ca ions on Ph kinase which converts Ph *b* to Ph *a*. Taken together, from the beginning of his strategy, Krebs did not consider the determination of the concentration of Ca ions required for activation of Ph kinase and also the detection of the reversibility in this assay model. This was the reason why Meyer *et al.*[81] were unable to find the correct answer in 1964. Thus, I realized that Krebs did not understand the modern theory of the regulatory mechanism of muscle contraction by low concentrations of Ca ions.

6.2. Our assay model and reversible activation of Ph kinase by low concentrations of Ca ions. First, I tried to answer the two questions that Professor Ebashi had suggested to me, namely, [Question 1] whether low concentrations of Ca ions activate Ph kinase and [Question 2] whether the activation is reversible. These are designated as our first working hypotheses in this article.

In our following experiments, I adopted a modified Krebs' model. I completely omitted his [Step 1], which was the most important step for Krebs' group,[84] and thus our initial and main reaction was [Step 2]. In this reaction, we used a metal ion buffer with EGTA to keep the free Ca ion concentration constant. The Ca ion concentration in the reaction mixture varied with each test tube, which ranged between 10^{-7} and 10^{-4} M. Our reaction mixture contained Ph kinase, Ph *b*, Mg-ATP, Ca ions at a specific concentration, and a pH buffer to keep the reaction mixture at pH 6.8.[85] After incubation for the main reaction, an aliquot was diluted and then assayed for Ph *a*, this process corresponds to Krebs' [Step 3].

In this assay model, we were able to determine the effect of various concentrations of Ca ions in the reaction mixture for the conversion of Ph *b* to Ph *a*. We considered that by changing the concentration of Ca ions in the main reaction, we can mimic the Ph kinase reaction *in vivo* where the concentration of Ca ions fluctuates.

In the fall of 1966, we obtained evidence for our first working hypotheses and were ready to publish our findings. However, Professor Ebashi did not decide whether to publish our data immediately. A few months elapsed before the submission of the first paper. At last, in April 1967, we published a short paper[85] showing that low concentrations of Ca ions that are released from SR could reversibly activate Ph kinase. Although the full paper[86] for these observations had already been written in 1968, the publication of this paper was delayed until 1972, for the reason described below. In the present review, I refer to the figures that were published in the full paper, because these are more suitable for detailed analysis and discussion than those of the short paper, although the data published in these two papers are essentially the same.

[Answer to Question 1: the range of concentration of Ca ions that activate Ph kinase] During the relaxation–contraction cycle, the concentration of free Ca ions in cytoplasm changes, ranging roughly from 10^{-7} to 10^{-5} M or slightly more widely, according to various reports. Thus, Ph kinase should show a distinct activity corresponding to each concentration of Ca ions within this range.

The Ca ion concentration vs. Ph kinase activity curve for rabbit muscle was sigmoid (Fig. 3). This curve roughly coincided with the Ca ion concentration vs. muscle tension curve for the skinned frog muscle fiber obtained by Ebashi and Endo,[2] for example. This means that the percentage of Ph kinase activity increases when muscles more strongly contract. Roughly, in the presence of Ca ions that provide a plateau tension, the percentage of Ph kinase also reaches a plateau. This means that when more ATP is required for contraction, the percentage of Ph *a* activity increases and a larger amount of glycogen is degraded. However, Ph kinase was slightly activated at 10^{-8} to 10^{-7} M Ca ions, at which contraction did not occur as shown by Ebashi and Endo.[2] This suggests that even when measurable tension does not occur, glycogenolysis must take place albeit slightly. This may be compatible with the observation by Solandt[87] in 1936: when muscle fibers were immersed in slightly K ion-rich Ringer solution, lactate was produced despite the fact that appreciable tension was not measured.

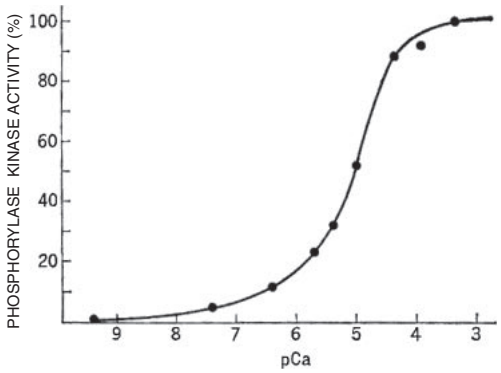

Fig. 3. Relationship between concentration of free Ca ions and Ph kinase activity (Ozawa, 1972[86]). Reaction mixtures of Ph kinase assay contained as the final concentrations: 8×10^3 units/ml Ph *b*, 4 mM ATP, 6 mM Mg Cl_2, 1.3 mg/ml Ph kinase, 30 mM *β*-glycerophosphate, 60 mM Tris, 3 mM EGTA (pH 6.8) and a specified concentration of Ca ions. This mixture was incubated at 30 °C, for 5 min. An aliquot was taken and diluted with 21 times dilution with ice cold cysteine (30 mM)-*β*-glycerophosphate (40 mM) buffer (pH 6.8). This was assayed for Ph *a*. The activities of Ph kinase were expressed by relative values, *i.e.*, percent of that in the presence of 4×10^{-4} M Ca ions; the latter activity converted 6.8 units of Ph *b* to Ph *a* per 5 min in the assay medium employed. The vertical line depicts Ph kinase activity. The horizontal line depicts the concentration of free Ca ions in the reaction mixture of Ph kinase.

[Answer to Question 2: Reversibility of Ph kinase activation by Ca ions] Ph kinase must be reversibly activated *in vivo* with the increase and decrease in the concentration of Ca ions during the contraction–relaxation cycle. We considered that this can be mimicked by the artificial change in the concentration of free Ca ions in our main reaction during incubation. As shown in Fig. 4, Ph kinase showed high activity in the presence of a Ca ion-

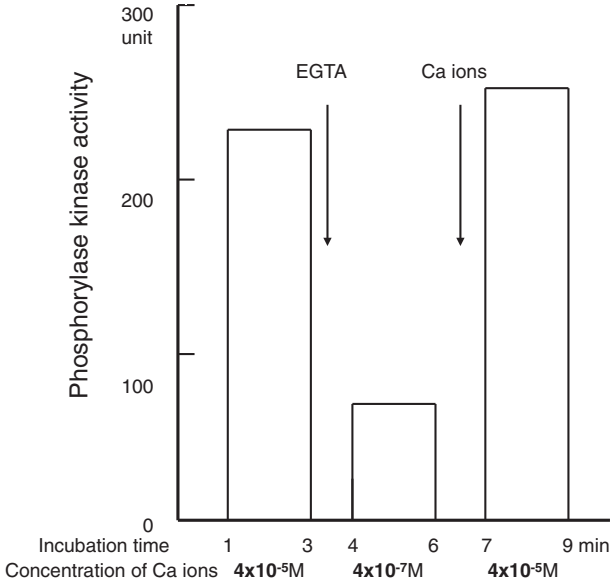

Fig. 4. Reversibility of activating effect of low concentrations of Ca ions on Ph kinase (Ozawa, 1972,[86] revised). The time course concerning full activating and reduced activating processes by addition and removal of free Ca ions of Ph kinase are shown. The kinase assay medium contained 4×10^{-5} M Ca ions at the start of reaction. After the three aliquots of the reaction mixture were removed every minute to determine the activity of Ph kinase, EGTA solution was added to the assay medium to lower the concentration of Ca ions down to 4×10^{-7} M. Three aliquots of the reaction mixture were removed again every minute. Then, $CaCl_2$ solution was added to the reaction mixture up to 4×10^{-5} M. To neutralize the hydrogen ion which was produced as a result of chelation of Ca ions, calculated amount of NaOH was added to the reaction mixture. To minimize the dilution effect, an appropriate amount of ATP with Mg was added to the reaction mixture. The activities thus obtained were corrected for the dilution effects. The vertical axis denotes the activity of Ph kinase. The horizontal axis shows the time of incubation. Concentrations of free Ca ions in the incubation mixture of Ph kinase are shown at the bottom of the horizontal axis.

concentration of 4×10^{-5} M. Then, the activity decreased when the concentration of Ca ions markedly decreased to 4×10^{-7} M. Furthermore, it again increased with the increase in Ca ion concentration to 4×10^{-5} M. Thus, the activity of Ph kinase reversibly changes with the change in Ca ion concentration.

On the basis of the results of the two above-mentioned experiments, our first working hypotheses were realized. It was thus concluded that Ca ions released from SR on excitation–contraction coupling simultaneously regulate contractile elements and Ph kinase (Fig. 2).

6.3. Response of Krebs to our works. In the summer of 1967, after our first short paper was published, Krebs came to Tokyo to attend the International Congress of Biochemistry, and visited Ebashi's laboratory to discuss this problem. Krebs honestly told us that he was sticking to the role of KAF and Ebashi explained the regulation of muscle contraction by low concentrations of Ca ions and the metal ion buffer, stressing the difference in the Ca ion binding effect between EGTA and EDTA in the presence of Mg ions at mM-level.

In 1971, Brostrom *et al.*[88] published a paper showing that low concentrations of Ca ions reversibly activate Ph kinase, confirming our results. They further showed that Ca ions bound to their Ph kinase fraction. In the introduction of their paper, they claimed that they had already observed the reversibility before us in 1964.[81] In this paper, they wrote that the reversibility of the activation of Ph kinase is essential when considering their process as the physiological regulatory system. However, what they mainly showed was the irreversible activation of Ph kinase in the presence of high concentrations of Ca ions and KAF. There were supplementary results showing that at pH 8.2, the "activity of non-activated Ph kinase" decreased following the addition of EDTA or EGTA and increased again following the addition of various divalent metal ions including Ca ions. Regarding this finding, they only wrote, "The relationship of a role for Ca ions in kinase reaction itself to the role for Ca ions in kinase activation as described in this paper is not clear, although the existence of such a relationship is an attractive unifying possibility." At that time, they did not understand their own results. Thereafter, they did not mention this process until our works were published. There were serious problems with their results that prevented for us from considering them represent physiological reactions. (1) Meyer *et al.*[81] conducted such experiments only at pH 8.2. However, pH of the living cytoplasm remains around neutral pH, and does not increase to such a high level. This result does not represent a physiological phenomenon. Furthermore, they did not explain what this activity means. (2) They did not pay any attention to the effective free concentrations of Ca ions for activating Ph kinase in their experimental system. Naturally, they did not consider whether the low concentrations of Ca ions at which muscle contraction is induced can activate Ph kinase. Thus, the claims of Brostrom *et al.*[88] are biologically unreasonable and are not acceptable. In the early 1970s, some other researchers accepted our concepts.[3),89),90)] Finally in 1979, Krebs wrote as follows in his review[1)] citing our paper:[85)] "Phosphorylase

kinase was recognized as having Ca ions requirement in 1964.[81] Later, this requirement was quantified, and it was shown that stimulation of the enzyme by Ca ions occurs within the physiological range of metal ion concentrations.[85]" With these words, Krebs at last accepted the validity of our work and agreed that their works did not represent physiological reactions and did not give the final answer to the mechanism of activation of glycogenolysis upon muscle contraction. However, our works were overlooked or ignored by the authors of books on signal transduction decades later.[91),92]

In 1968, Huston and Krebs[93] showed that KAF is a Ca-requiring nonspecific protease and finally abandoned the working hypothesis of KAF as the physiological kinase activating factor. This protease was later renamed calpain.

6.4. Is phosphorylase kinase sufficiently activated by Ca ions from SR for phosphorylase conversion upon muscle contraction? With the progress of our works, I realized that our answers to [Questions 1 and 2] could explain that Ca ions released from SR can reversibly activate Ph kinase. However, our results do not answer [Question 3], whether Ca ions at above-stated concentration can quantitatively sufficiently activate Ph kinase to actually convert Ph *b* to Ph *a* at the rate of the reaction that occurs *in vivo* upon muscle contraction. If it is not the case, Ca ions released from SR cannot be the physiological regulatory factor for glycogenolysis upon contraction. For example, if the total activity of Ph kinase in the presence of the concentration of Ca ions that gives the maximum tension of muscle fiber reaches only 1% of the activity to convert Ph *b* to Ph *a* *in vivo*, it is not sufficient for the coupling between glycogenolysis and contraction. Thus, I considered that [Question 3] must be surveyed to determine the reversible activation of Ph kinase by low concentrations of Ca ions is physiological.

Pieces of rabbit muscle were homogenized and centrifuged, and the resulting precipitate was again homogenized and centrifuged.[94] After this procedure was repeated three times, the supernatant was pooled. The pooled sample was assayed for phosphorylase in the presence of 4×10^{-3} M AMP that activates Ph *b* and for Ph kinase activity in the presence of 4×10^{-5} M Ca ions. The unit of Ph kinase is defined as [Ph units converted from Ph *b* to Ph *a* per sec]. Therefore, the ratio of the total activity of phosphorylase/the total activity of Ph kinase gives the time expressed in sec required for the conversion of total Ph *b* to Ph *a*. The total activities of phosphorylase and Ph kinase we obtained were 9,160 units/g muscle and 1,490 units/g muscle, respectively (the mean of muscles from six different rabbits). Thus, the ratio was 6.1 sec in rabbit muscle. The time needed for 80% activation of tetanized frog muscle obtained by Danforth and Helmreich[82] was 2.5 sec, as determined from their data. Thus, our conversion time was roughly twice theirs. Taking into consideration that the time we obtained was calculated from the data obtained *in vitro* under enzyme assay conditions and theirs were those derived *in vivo* under physiological conditions, I considered that these data were roughly compatible.

On the basis of our answers to [Questions 1, 2 and 3], we concluded that low concentrations of Ca ions released from SR can actually regulate glycogenolysis upon muscle contraction. This relation may be called "excitation–metabolism coupling" after the term "excitation–contraction coupling".

Finally, on the basis of our studies and those made previously by others, we further claimed that an ATP-consuming system, *i.e.*, the contractile system, and an ATP-producing system, *i.e.*, the glycogenolysis system, are simultaneously controlled by a single substance, namely, Ca ions (Fig. 2, box). This predicted the possibility that a one-sided activation of these two systems in muscles is excluded. In this way, muscle fibers can contract continuously, for a long time.

6.5. Relationship between cyclic AMP and Ca ions in Ph kinase. Our next question [Question 4] was whether there is any relationship between the activation of Ph kinase by cyclic AMP and that by low concentrations of Ca ions.[94] At the time when Krebs' group was studying regulation of Ph kinase, the activation mechanism by high concentrations of Ca ions with KAF and that by cyclic AMP were separately studied. They did not consider interaction of the cAMP and Ca ion regulation systems.

We assayed Ph kinase from cardiac and skeletal muscles in the presence of low concentrations of Ca ions and cyclic AMP[86),94] together. We found that cyclic AMP did not activate Ph kinase in the absence of Ca ions at the concentrations required for Ph kinase activation without cyclic AMP (Fig. 5). However, in the presence of low concentrations of Ca ions, Ph kinase activity was enhanced by cyclic AMP. Thus, it became clear that what is essential for the activation of Ph kinase is Ca ions, and cyclic AMP only promotes this process.

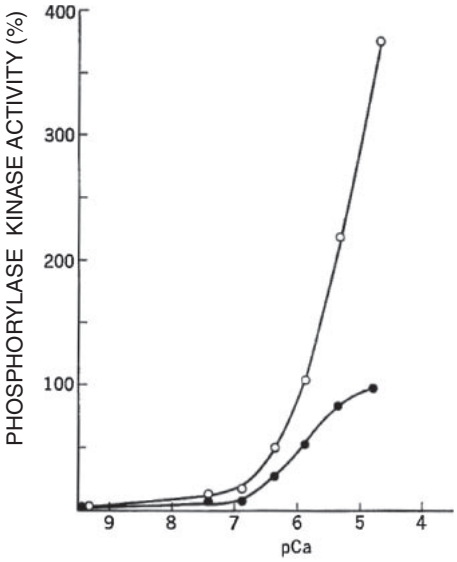

Fig. 5. Relationship between the activity of phosphorylase kinase from cardiac muscle and the concentrations of free Ca ions in the presence and absence of cyclic AMP (Ozawa, 1972[86]). Closed circles indicate the activities without cyclic AMP and open circles indicate those with 3×10^{-6} M cyclic AMP. Note that the activity is almost zero in the presence or absence of cAMP, in the presence of Ca ions between 10^{-9} and 10^{-7} M, at which contraction does not occur. In the presence of Ca ions between 10^{-6} and 10^{-5} M, Ph kinase activity was potentiated by cyclic AMP.

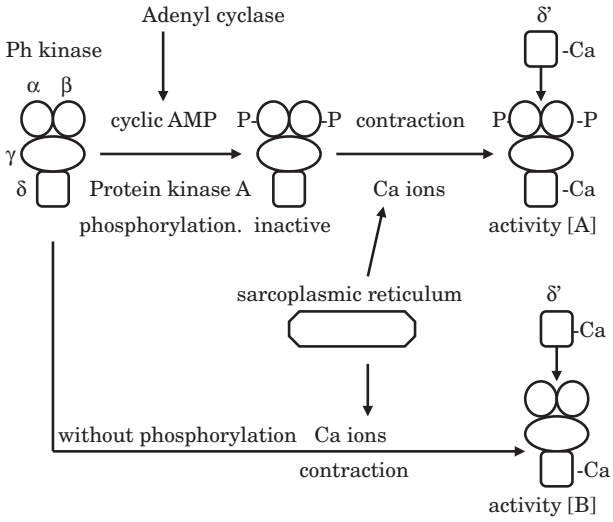

Fig. 6. Relationship of cyclic AMP-dependent phosphorylation of Ph kinase with activation by Ca ions. Greek letters (α, β, γ, δ, and δ') designate the names of subunits of Ph kinase. Further details, see text Sections 6.5 and 7.1.2.

Before our works were published,[94] cyclic AMP-dependent protein kinase[95] (protein kinase A) had not been reported. Retrospectively considered, in our assay system, it can be assumed that Ph kinase was phosphorylated by protein kinase A in the presence of cyclic AMP that was contaminating the Ph kinase samples at the same time when Ph kinase catalyzed the conversion of Ph *b* to Ph *a*.

After the higher structure of Ph kinase were revealed and the substrates of the protein kinase A was discovered (see Section 7), our data were more clearly interpreted as follows (Fig. 6): With or without phosphorylation of α- and β-subunits by protein kinase A, Ph kinase is not activated in the absence of low concentration of Ca ions that bind to δ-subunit. However, in the presence of the Ca ions, phosphorylated Ph kinase [A] show higher activity than non-phosphorylated Ph kinase [B]. Thus, cyclic AMP-protein kinase A system potentiates the effect of Ca ions on the Ph kinase activity, *via* phosphorylation of Ph kinase, although the system alone does not activates Ph kinase.

It is sometimes illustrated in textbooks that in the phosphorylase activation cascade, activation with cyclic AMP is located upstream of activation with low concentrations of Ca ions. This is not correct, because phosphorylation by protein kinase A is not required for inducing the Ph *b*-to-Ph *a* conversion by low concentrations of Ca ions.

Even today, there are some who ask why Ph kinase is regulated by both cyclic AMP and Ca ions. It is pharmacologically clear that when epinephrine activates adenyl cyclase, the resulting cyclic AMP may accumulate in the cytoplasm, which, in turn, activates protein kinase A to phosphorylate Ph kinase. However, phosphorylated Ph kinase does not convert from Ph *b* to Ph *a* in the absence of Ca ions. That is, without contraction during which the muscle consumes ATP, glycogenolysis does not occur even when epinephrine is present. If a stronger contraction occurs in the presence of epinephrine, as in the case of cardiac muscle, the breakdown of more glycogen is required to produce more ATP. This is the reason why Ph kinase is regulated as described above.

In addition to the above-described findings obtained using skeletal and cardiac muscles, I determined that Ph kinases from the chick gizzard smooth muscle[86] and guinea pig brain[96] were likewise activated by low concentrations of Ca ions.

6.6. Sudden stop of our works. There were still some additional questions that needed to be answered, but our experiment was abruptly finished owing to the shutdown of Ebashi's laboratory by

students in 1968. Thus, I had to stop my work and could only write my full paper.

Leaving my manuscript[86] to Professor Ebashi for publication, I moved to a laboratory in the United States in 1969, where I worked on muscle development. It was unfortunate for me that our full paper was published only in 1972, because of the turmoil in the university.

More than 40 years has elapsed since the interruption of our study. No new concepts on the physiological mechanism of glycogenolysis upon muscle contraction have been reported, although there has been progress on the biochemistry of Ph kinase and some of its applications. Therefore, I consider that many researchers have accepted that this fundamental question was practically solved by our works (see Needham[3] p. 431 and p. 446). A few years after the publication of our papers, Krebs left the project on Ph kinase, and he and Fischer devoted themselves to the study of protein phosphorylation of various systems.

7. Further studies related to phosphorylase kinase and Ca ions

Since I left research on phosphorylase kinase, the roles of low concentrations of Ca ions have been very widely appreciated. Today, the research fields on Ca ions have tremendously developed in modern molecular and cell biology. It is absolutely impossible for me to summarize all these fields related to the regulatory function of Ca ions. Here, I would like to very briefly mention the development of subjects closely related to our works concerning the roles of Ca ions and phosphorylase in order to identify the position of our works in this enormous field of the investigation of signal transduction.

7.1. Ca-ion-related processes, including Ca-dependent signal transduction.

7.1.1. Designation of Ca ions as a second messenger. Hidaka and Kakiuchi[97] in 1981 compactly described the beginning of studies of regulation of enzymes by Ca ions as follows: "The first report that described that Ca ions serve to regulate the activities of intracellular enzymes was the "activation of muscle phosphorylase kinase" by Ozawa[86] in 1967. After this work, the discovery of Ca-dependent phosphodiesterase by Kakiuchi[98] followed in the spring of 1970. This discovery was confirmed by Canadians, Teo and Wang,[99] in 1973. Later on, many intracellular enzymes were found to be regulated by Ca ions. Ever since, Ca ions have been known as the intracellular second messenger" (see Section 3.2 for the original meaning of second messenger, and see Section 4.4 for intracellular enzyme regulated by Ca ions). The regulation of muscle contraction by Ca ions was thoroughly examined from various viewpoints. It is unfair that the role of Ca ions in muscle contraction has only supplementally been described or omitted in the current textbooks of signal transduction.[92),100]

From the viewpoint of modern cell biology (Table 1), Ph kinase, which was discovered by Fischer and Krebs[73] in 1955, was the first protein kinase that was observed to cause a change in the function of a phosphorylated protein, and the process of its reversible activation by low concentrations of Ca ions that we described in 1967 turned out to be the first case of a Ca-dependent process of protein kinase.[85] With our discovery, the Ca-dependent protein phosphorylation process was first introduced into the signal transduction study. However, we did not directly contribute to the following development.

7.1.2. Calmodulin and Ph kinase. In 1970, Kakiuchi and Yamazaki[98] showed that low concentrations of Ca ions activate cyclic mononucleotide phosphodiesterase as described above. In the same year, Cheung[101] and Kakiuchi *et al.*[102] almost simultaneously and independently reported the presence of a heat-stable protein factor that was coprepared from phosphodiesterase. Kakiuchi *et al.* reported this time that the addition of this factor together with Ca ions to purified diesterase resulted in the activation of the enzyme. In 1973, this protein was fairly well purified by Wang's group.[99] In the same year, they[99] and Kakiuchi *et al.*[102] independently reported that this protein is a factor activating diesterase in the presence of Ca ions. This was the discovery of calmodulin that was later found to be a Ca-dependent regulating factor for a number of intracellular enzymes. Because calmodulin is usually not a subunit of an enzyme but binds to the enzyme only when Ca ions bind to it, Kakiuchi *et al.*[102] suggested the following scheme.

$$[\text{enzyme}]_{\text{inactive}} + \text{calmodulin} + \text{Ca} \rightarrow [\text{enzyme-(calmodulin-Ca)}]_{\text{active}}$$

Calmodulin has molecular weight of 17 kDa and forms a family together with troponin C (for monograph[97),103]).

Owing to the progress of purification methods of Ph kinase and of the establishment of the SDS-gel electrophoresis method, Ph kinase was found to be composed of three subunits, namely, α-, β-, and

γ-subunits, by Hayakawa *et al.* of Krebs group[104] and Cohen[89] independently, in 1973 (Fig. 6). It was found by these researchers that the α- and β-subunits are phosphorylated by protein kinase A, whereas the γ-subunit was identified to be the catalytically active subunit of Ph kinase by Skuster *et al.*[105] in 1980. It is noteworthy that in 1978, Cohen *et al.*[106] discovered the δ-subunit and identified as calmodulin. However, we must note that the δ-subunit is associated with other subunits even in the absence of Ca ions. The structure of Ph kinase was determined to be $(\alpha\beta\gamma\delta\text{-subunits})_4$ and the molecular weight was 1,500 kDa. Picton *et al.*[107] further reported that an additional calmodulin or the δ'-subunit binds to both α and β subunits in the presence of Ca ions and its catalytic activity was further increased. The δ'-subunit was found to be replaced by troponin C.

Today, in the presence of Ca ions calmodulin is known to be associated with various enzymes, channels, and other cell structures related to cell signaling. Even adenyl cyclase is regulated by the Ca-calmodulin system.

7.1.3. Ca-dependent protein phosphorylation. In 1977, Takai *et al.* of Nishizuka's group[108] discovered a protein kinase from the brain different from protein kinase A. In 1979, Takai *et al.*[109] further found that this enzyme was activated by a minute amount of unsaturated diacylglycerol and phosphatidylserine in the presence of 10–100 µM Ca ions, and it was named protein kinase C. For the regulation of the activity of this enzyme, calmodulin was not required. In the same year, Takai *et al.*[110] progressed their observation and indicated the possibility that unsaturated diacetylglycerol which may be derived from phosphatidylinositol turnover provoked by various extracellular stimulators, acts as a messenger for activating the protein kinase C and various phospholipids seem to play a role cooperatively in this unique receptor mechanism. Thus, this study opened the door for connection of between Ca-dependent signal transduction and lipid metabolism.

Over the years, it became clear that there are at least 12 types of protein kinase C.[111] These enzymes are very widely distributed in various species and organs. Their functions vary, and are related to various phenomena, such as tumor formation, inflammation, embryonic development, pain sensation, visual sensation, and long-term memory. Their mechanisms of function are complicated but have been well documented in many cases (see monograph[111]).

7.1.4. KAF is a protease calpain. KAF that was once assumed by Krebs to be essential for the activation of Ph kinase turned out to be a protease activated by Ca ions at the mM level,[93] that was originally discovered in brain by Guroff[112] in 1964. As stated above, the intracellular concentration of Ca ions cannot be this high level under normal conditions. Some suspected the enzyme to be a postmortem scavenger. However, in 1978, Sandoval and Weber[113] discovered a protease that is activated by Ca ions at the µM level. In the same year, Nishiura *et al.*[114] of Murachi's group showed the presence of an endogenous inhibitor of this enzyme. Afterwards, these proteases are termed μ-calpain and m-calpain, depending on the concentration of Ca ions necessary for activation. In 1984, Ohno *et al.*[115] of Koichi Suzuki's group revealed the primary structure of the calpain catalytic subunits and calpain studies turned out toward structure–function analysis. For further details, see a recent comprehensive review.[116]

Calpain present in muscles suffering from muscular dystrophy has been implicated in the digestion of muscular proteins resulting in muscular atrophy. I have recently discussed this problem in another review appeared in this journal.[117] In addition, the mutation of calpain-3[118] was found to be responsible for limb-girdle muscular dystrophy.

In addition to those stated above, there are many biomechanisms that are regulated by Ca ions (for monograph[119]).

7.2. Ph-kinase-related processes.

7.2.1. Ph kinase converts glycogen synthase from active form to inactive form. Ph kinase promotes glycogenolysis, converting Ph *b* to Ph *a*; at the same time, it suppresses glycogen synthesis by converting active glycogen synthase (GS) *a* to inactive GS *b*.[120] At the molecular level, in contrast to phosphorylase, when GS is phosphorylated, it loses its activity, whereas when it is dephosphorylated, it acquires the activity to synthesize glycogen. Thus, Ph kinase activated by Ca ions simultaneously phosphorylates Ph *b* and GS *a*, inducing the breakdown of glycogen by activating Ph *b* and the blocking synthesis of glycogen by inactivating GS *a*, respectively. Some claim that some other protein kinases, such as protein kinase A, are also involved in this process.[121]

7.2.2. Malignant hyperthermia. This genetic disease is characterized by attacks of cramp of whole muscles and very high fever that occur when the patients are anesthetized with halothane. This is mostly due to the mutation of the ryanodine receptor (the Ca ion channel of SR) gene or *RyR1*, giving rise

to an increased susceptibility to the anesthetics (for review[122]). In this disease, a specific Ca-ion-releasing mechanism, Ca-induced Ca release (for review[123]), is apt to work,[124] and the high concentration of cytoplasmic Ca ions is sustained following the administration of anesthetics, resulting in continuous contraction. In addition to the contraction, Ph kinase is activated by Ca ions, which results in the continuous production of ATP that is a prerequisite for continuous heat production, one of the essential symptoms of this disease.

7.2.3. Phosphorylase and Ph kinase gene mutations. The mutations of phosphorylase and Ph kinase have been discovered in humans. These mutations cause muscle rigor upon severe movement. This suggests that in extreme cases, glycogenolysis is not switched on following the release of Ca ions from SR, and only the contraction system is switched on, leaving glycogenolysis inactivated. It is assumed that ATP is depleted when muscle rigor occurs. For technical reasons in human muscle biopsy, ATP depletion has not been reported (for review[125]). However, it is conceivable that ATP depletion is the actual cause of rigor in these diseases (see Section 2.2).

8. Epilogue

As described above there were two large research currents in muscle physiology (Table 1). The first current included the studies of glycogenolysis upon muscle contraction, which was studied since the later half of 19th century and the second current included the studies of the molecular mechanism of muscle contraction. These two large rivers flew at first slowly and quickly during the middle of 20th century. Thanks to the efforts of my many senior researchers, especially those of Krebs and Fischer and those of Ebashi and Weber, these two large rivers closely approached each other to form a very narrow isthmus.

In 1965, when I was a newcomer to science research, I stood at the isthmus. With the suggestions of Professor Ebashi, I could luckily construct a small canal to connect these rivers by showing that low concentrations of Ca ions that activate the contractile system simultaneously and reversibly activate Ph kinase. Since our works were published, this problem has essentially not been challenged further from the physiological viewpoint.

Later, with the advent of the new era of molecular and cell biology, our work was found to have another facet. Here, I would like to recapitulate that Ph kinase is the first protein kinase discovered by Krebs and Fischer and that regulation of the protein-phosphorylation by low concentrations of Ca ions was first described by us. In relation to the concept of Ca-regulated protein phosphorylation, the great discovery of calmodulin and that of protein kinase C were made within 10 years after our works. In the same period, Krebs and Fischer generalized the concept on protein phosphorylation as the essence of signal transduction.

It is my great honor to say that we could not only connect the two great rivers of muscle physiology, but also, as a result of the later progress of bioscience, construct a small path for concept of regulation by Ca ions to move from its birth place, the muscle physiology, to the much larger and growing research fields of "calcium biology" including signal transduction.

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