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Ca^{2+} in contractile processes

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The concept of Ca^{2+} regulation, first discovered and developed in muscle research, is historically surveyed. Ca^{2+} regulation mechanisms in actomyosin-dependent contractile processes are compared, emphasis being placed on the great diversity. The mode of action of Ca^{2+} is discussed with the examples of troponin and calmodulin, the most differentiated and conservative Ca^{2+}-receptor proteins, respectively.

1. Introduction

Ca^{2+} is now widely accepted as the most fundamental and crucial regulator of intracellular processes. This idea was born in the muscle field and developed almost exclusively in muscle research until recently (cf. refs 1 and 2).

The establishment of the essential role of Ca^{2+} not only in contractile processes but also in some metabolic pathways in the 1960s [1,3] tempted us to propose a general scheme for the activity of living organisms as indicated in fig. 1. That is, the activity is classified into three areas. One is to utilize various materials as the source of energy for maintaining the active state of life. Simply speaking, its main role is to produce ATP. The second is to produce its own copy, i.e., the field of genetics and developmental biology, primarily based on the strategy of protein synthesis. The third is to respond to the impulses or signals from the milieu exterior, resulting in, for instance, the excitation of nerve cells or the perception and thinking of the brain, where the primary steps are ionic processes at the membrane systems. Ca^{2+} plays a major role, alone or in collaboration with other factors, not only in eliciting so-called physiological function in its own area, but also in signal transmission or communication to the other two.

This scheme might not be so absurd even in

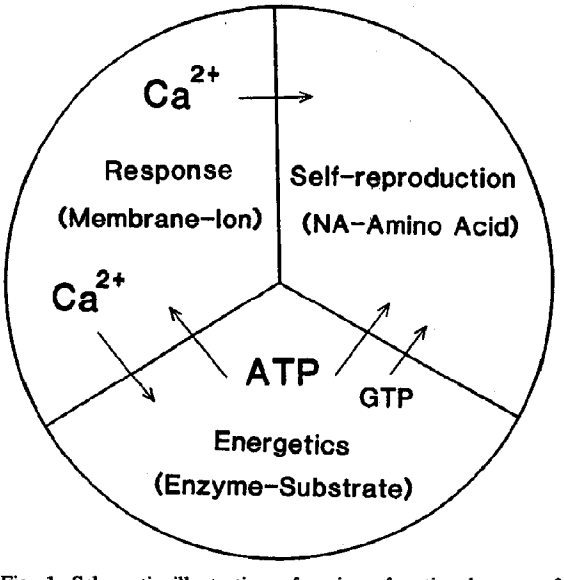

Fig. 1. Schematic illustration of various functional areas of living organisms and of the role of Ca^{2+} in linking between different functional areas. For explanation see the text (NA, nucleic acid; 'membrane' includes not only surface membrane but also intracellular membrane systems).

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view of the advanced knowledge at the present stage. The position of Ca^{2+} in developmental biology is being increasingly enhanced by numerous findings, although its mode of action is very complicated.

This important role of Ca^{2+} is based on the virtual absence of free Ca^{2+} in the cytoplasm, the difference between the inside and outside of cells being more than 10 000-fold, thus producing the highest electrochemical potential in living organisms. The energy of ATP seems to serve primarily in maintaining this potential, directly or through an Na^+ gradient. Since this feature can be observed even with primitive seaweeds, it was acquired by living organism during very early stages of evolution, perhaps at the same time as the cell was created. Thus, the evolution of life must be related to geophysics; the inorganic composition of seawater, especially that of Ca^{2+}, played a determining role in creating life.

2. Ca^{2+} in muscle

The essential role of Ca^{2+} in muscle contraction was suggested from experiments with living muscles in early 1940s, but was not accepted by those who pursued the mechanism of muscle contraction through studies on the actomyosin-ATP system, which did not seem to require Ca^{2+} (cf. ref. 1). One of the reasons why those workers could not recognize Ca^{2+} was that the Ca^{2+} concentration required for muscle contraction was unexpectedly low, such as 10^{-6} M [1,4–6] (fig. 2).

The next breakthrough in Ca^{2+} research was the discovery in the mid 1960s of troponin [7,8] (cf. refs 1 and 2), the first Ca^{2+}-receptor protein of physiological significance [9]. In spite of such concrete evidence, however, subsequent Ca^{2+} research was still confined to the muscle field. Demonstration a significant role of Ca^{2+} in fields other than that of muscle had to await the work of Kakiuchi and Yamazaki [10] in 1970 who observed the Ca^{2+} dependence of brain phosphodiesterase.

Nowadays, Ca^{2+} regulation is notable because of its extraordinary variety. Even if the matter is confined to actomyosin systems, four types of Ca^{2+} regulation can be enumerated (table 1). The troponin system operates in higher animals, but is also distributed in lower animals. Its isolation from lower animals had been hindered because of its susceptibility to proteolysis, but the development of effective protease inhibitors has made it much easier to detect troponin in lower species, even in molluscs [11] in which a myosin-linked system was found because of the 'absence' of troponin.

The myosin-linked system [12,13] is distributed in animals lower than chordates (Deuterostomia) or the horseshoe crab (Protostomia). Regulation in smooth muscle and slime mold will be discussed below.

The mode of regulation varies from tissue to tissue. Troponin regulation is notable because of its de-repressive nature [1], which is suited to the rapid and exactly reproducible contraction of skeletal muscle, whereas smooth muscle is simply of the activation type [2]. Ca^{2+}-binding sites in these two systems reside in small molecules,

Table 1

Ca^{2+} regulation mechanisms in actomyosin-dependent contractile systems

Type	Ca^{2+}-receptor protein	Location of Ca^{2+} receptor [a]	State of Ca^{2+} receptor	Role of Ca^{2+}
Troponin	troponin C	actin (troponin I-tropomyosin)	firmly bound	de-repression
Myosin-linked	myosin [b]	myosin		(de-repression)
Smooth muscle	calmodulin	actin (MLCK)	flip-flopping	activation
	calmodulin	actin (leiotonin)	flip-flopping	activation
Slime mold	14 kDa light chain	myosin	firmly bound	repression
	14 kDa light chain	actin	loosely bound	repression

[a] Proteins in parentheses are those to which the Ca^{2+}-receptor proteins are directly bound.

[b] If one of its light chains is removed by EDTA, Ca^{2+} sensitivity and Ca^{2+}-binding capability are lost, but this does not mean that the light chain is the Ca^{2+}-binding site itself, which has not yet been precisely allocated.

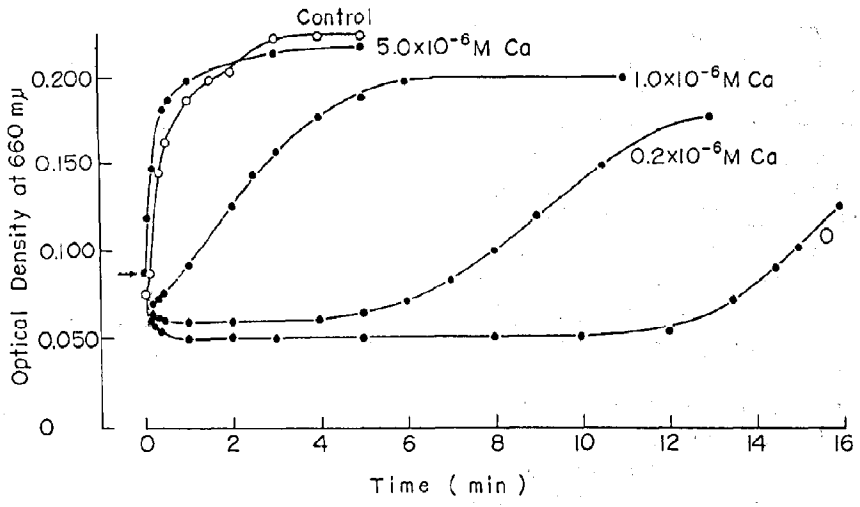

Fig. 2. Effects of Ca^{2+} on Ca^{2+}-free natural actomyosin of skeletal muscle. Abscissa indicates the degree of superprecipitation, which represents contraction in vitro. Note that 2×10^{-7} M Ca^{2+} shows a definite effect and that less than 5×10^{-6} M Ca^{2+} is enough to produce the full activity (quoted from ref. 6). This experiment was carried out by adding net concentrations of $CaCl_2$ corresponding to the Ca^{2+} concentrations as indicated, without using Ca^{2+} buffer; for this purpose, all components in the reaction mixtures, i.e., water, reagents and actomyosin, were subjected to various procedures to remove contaminated Ca^{2+} as far as possible.

troponin C, a subunit of troponin, and calmodulin, respectively, but their modes of action are entirely different [2]. Troponin C never detaches from the parent molecule; although it is composed of three subunits, troponin behaves almost as a single molecule like myosin, which is also a complex of two sets of three subunits. In sharp contrast, calmodulin detaches from the parent protein in the absence of Ca^{2+}; this has been expressed as flip-flop type regulation [14], which bestows complex properties on this simple protein (actually, calmodulin is often retained by its target protein even in the absence of Ca^{2+}, but in this case the affinity is very much reduced, so that the concept of flip-flop regulation virtually operates in most cases).

3. Ca^{2+} regulation in slime mold

The slime mold, *Physarum polycephalum*, is a miraculous organism. The ordinary i.e., plasmodial, type is somewhat similar to fungi, being undoubtedly a plant, but owing to the change in environment it converts into amoeboid type, an animal-like creature. While the former carries out vigorous cytoplasmic streaming like some plants, the latter performs amoeboid movement as expressed by its name.

Plasmodial slime mold contains abundant Ca^{2+}-dependent apyrase, which had previously prevented scientists from determining the true nature of its actomyosin system (the function of this apyrase has not yet been revealed). Eventually, Kohama et al. [15] succeeded in isolating natural actomyosin free of apyrase. Surprisingly, Ca^{2+} was shown to inhibit its contractility.

In spite of the diversity in the mode of action of Ca^{2+} from tissue to tissue, the final consequence brought about by Ca^{2+} had been shown to be the activation of contractile processes; this had been tacitly considered as inherent in the nature of Ca^{2+}. This traditional belief was completely disproved by the above finding. The inhibitory nature of Ca^{2+} also holds true for the actomyosin system of the amoeboid type slime mold (this does not mean that the actomyosin system underlying amoeboid movement in other organisms is inhibited by Ca^{2+}) [6,17].

The next question concerns the identity of the protein which is the Ca^{2+} receptor. It was revealed that the 14 kDa light chain of myosin, the smaller of the two light chains, was responsible for this

inhibition [17]. When *Plasmodium* is converted into the amoebal type, the heavy chain and the other light chain of myosin are also converted into amoebal types, respectively, but the 14 kDa light chain is the same for both types. The 14 kDa light chain is not homologous to the so-called regulatory light chains, viz., EDTA light chain of molluscan and phosphorylatable light chain of skeletal and smooth muscles, but is akin to the so-called alkali light chain of skeletal muscle [17]. This is an interesting finding with a deep evolutionary implication.

Another important aspect of slime mold regulation is that the 14 kDa light chain is also present in the cytoplasm and seems to exert its inhibitory action through actin [18]. Thus, slime mold also undergoes an actin-linked regulation, but it should be noted that virtually no tropomyosin is present in slime mold, indicating that the mechanism of actin-linked regulation in slime mold is very much different from that of the troponin-tropomyosin system.

Although not directly related to the subject of this article, we must refer to the recent revolutionary discovery of Yanagida et al. [19]. Summarizing: (1) under minimum load, the myosin head travels a distance of more than 10 actin molecules on consumption of only one ATP molecule; (2) under this condition, the dislocation of myosin along the actin rail can be more than 10-times faster than the maximum speed of muscle contraction. The latter finding has removed the barrier which had existed between muscle contraction and cytoplasmic streaming. The position of slime mold, which can carry out both cytoplasmic streaming and amoeboid movement, will thus become more crucial in future studies on cell motility.

4. Ca^{2+} regulation in smooth muscle

As stated above, the activation of the actin-myosin-ATP system of smooth muscle requires the cooperation of some other factor(s). The majority of smooth muscle researchers believe that this factor is no other than the Ca^{2+}-dependent myosin light chain kinase (MLCK) (cf. ref. 20). On the other hand, a minor group has been claiming that an actin-linked factor, called leiotonin, should play the principal role in activating the actomyosin system [2,19].

In the following, a few examples of evidence against the MLCK concept will be listed [21]:

(i) Bovine stomach and aorta contain 155 and 130 kDa components that show specific MLCK activity (K-activity), but the former exhibits about 10-times higher actomyosin-activating activity (L-activity) than the latter in comparison with their K-activities, respectively

(ii) Treatment of pure and crude 155 kDa component of stomach with a low concentration of trypsin abolishes most of its L-activity without affecting its K-activity.

(iii) The Michaelis constant of L-activity of chicken gizzard natural actomyosin (10 μM) is much smaller than that of its K-activity (0.2 mM or more); this means that at low concentrations of ATP, say, 20 μM, contraction takes place without phosphorylation.

It is certainly true that isolated phosphorylated gizzard myosin is vigorously activated by actin without Ca^{2+}, but the ATPase activity under this condition is about one-fifth that of skeletal actomyosin; this is very high compared with that of natural actomyosin. Bárány [22] has already shown that the ATPase activity of natural actomyosin of the uterus is about one-hundredth that of rat fast skeletal muscle. This is consistent with the low oxygen consumption of living smooth muscle in the contracting state (cf. ref. 23). On the other hand, isolated fresh smooth muscle myosin, which is soluble even at an ionic strength of 0.06 or less, soon loses its solubility in a day or so (in the state of natural actomyosin, this high solubility of myosin is retained for long periods). Phosphorylated myosin is insoluble at low ionic strength and dephosphorylation does not restore its solubility.

Our tentative conclusion is that the 155 kDa component is leiotonin itself, though we have not yet succeeded in preparing a K-activity-free preparation, and that the 130 kDa component is a genuine form of MLCK; perhaps the 130 kDa component of gizzard possesses the properties of both the 155- and 130 kDa components (the 80 kDa component, reported as gizzard leiotonin,

was a proteolytic product of the 130 kDa component).

5. Mode of action of calmodulin

The discoveries of troponin as the Ca^{2+}-receptor protein [8,9] and Ca^{2+} activation of phosphorylase *b* kinase [3] persuaded Kakiuchi and co-workers to look for an enzyme in brain that is activated by Ca^{2+}. Eventually they found that phosphodiesterase itself, on which they had been working for many years, was actually Ca^{2+} dependent [12] and that this activation was mediated by a protein factor [24,25]. Almost at the same time, Cheung [26] obtained from a snake venom a factor that stimulates phosphodiesterase. In this way, both scientists were the discoverers of calmodulin (CaM), but we should not forget the effort of Teo and Wang (27) in establishing CaM as a protein entity.

The fact that CaM plays a crucial role in smooth muscle regulation was demonstrated by Hartshorne's group [28]. Since smooth muscle contraction is regulated by Ca^{2+} in a very delicate manner, the following comments also have a very important relevance to the understanding of smooth muscle physiology.

6. How does CaM manage Ca^{2+} regulation by its low affinity for Ca^{2+}?

CaM has four Ca^{2+}-binding sites and the occupation of at least three seems necessary for its function. Its binding constant is unexpectedly low, 1.2×10^5 M^{-1} [29]. Among the four binding sites there is little cooperation. If anything, it is not physiologically important [30].

As is well known Ca^{2+} regulation in cells begins at a Ca^{2+} concentration around 2×10^{-7} M and becomes almost saturated at $3\text{–}5 \times 10^{-6}$ M (see also fig. 2). Ca^{2+}-binding constants of troponin can fully cover physiological Ca^{2+} concentrations. The question then arises as to how CaM can regulate the intracellular processes by such a low affinity for Ca^{2+} as mentioned above. If Ca^{2+} must bind to three or four sites, the sensitivity of a CaM-including system to Ca^{2+}

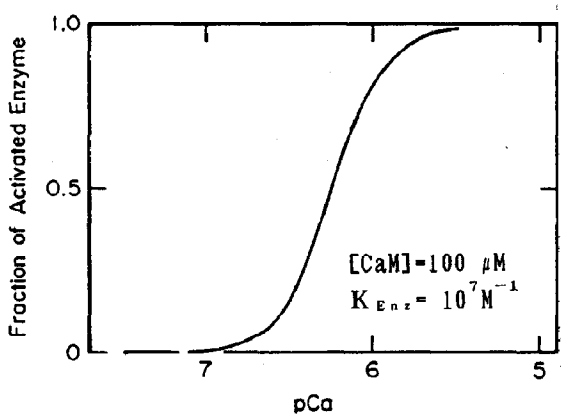

Fig. 3. Calculated relationship between p*Ca* and enzyme activity of CaM-involving system. For explanation see the text; for further details refer to ref. 30.

becomes worse than the average of the four Ca^{2+}-binding sites of CaM (cf. fig. 3 in ref. 30) and, therefore, cannot afford physiological requirements (we have discussed this problem in ref. 30 rather in detail; in the following only an outline of the discussion will be described).

The first necessary condition to overcome this difficulty is the high concentration of CaM. If the CaM concentration is much higher than the reciprocal of its Ca^{2+}-binding constant, a considerable amount of CaM can bind Ca^{2+} even at lower Ca^{2+} concentrations. The high CaM content of brain, gizzard and uterus may satisfy this necessary condition.

The formation of a Ca^{2+}-CaM complex by itself, however, could not settle the problem. A more crucial point is the affinity of the Ca^{2+}-CaM

Table 2

Affinities of ligands for CaM and apparent increases in affinity for Ca^2

Ligand	Affinity for CaM ($\times 10^6$) (M^{-1})	Increase in affinity for Ca^{2+} (-fold)
Phosphorylase *b* kinase	500	73
Phospho-diesterase	330	26
Myosin light chain kinase	67	14
Trifluoperazine	0.7	58

complex for enzymes or target proteins. This has been reported to be very high, viz., 10^7–10^{10} M^{-1} (table 2; cf. ref. 30). Even if we take a lower value, 10^7 M^{-1}, the calculated relationship between p*Ca* and the activity of the final complex satisfies the physiological requirement, provided such a high CaM concentration as 100 μM is present (fig. 3). This profile is in accord with those of many reactions involving CaM, exhibiting a fairly high cooperativity. If the affinity of Ca-CaM for enzyme is much stronger, even lower CaM concentrations will give a similar profile.

The upper three ligands in table 2 show a rather close relationship between the apparent increase in affinity of CaM for Ca^{2+} and the affinity of CaM for ligand (cf. ref. 30). Hence, the apparent increase in binding constants of CaM for Ca^{2+} may largely be explained by this mechanism. However, a different type of increase in affinity for CaM should be taken into consideration in view of the effect of trifluoperazine (bottom in table 2), the increase being far more than that expected from its affinity for CaM. We must assume a conformational change in CaM that results in an increase in the affinity for Ca^{2+}. Since the inhibitory effect of trifluoperazine on CaM-related reactions appears to be due to its binding to the site of CaM where target proteins bind, the above findings strongly indicate that the ligands could enhance the affinity of CaM for Ca^{2+} by inducing a change in the conformation of CaM. Interestingly, trifluoperazine increases the Ca^{2+} sensitivity of skinned fibers of skeletal muscle concomitantly with increase in the affinity of troponin C for Ca^{2+} [31]; it appears that trifluoperazine binds to troponin C and induces its conformational change, without affecting the binding between troponin C and troponin I.

7. A brief summary of Ca^{2+} regulation by CaM

As a whole, CaM can actually regulate intracellular processes by its low affinity for Ca^{2+}. Summarizing the above discussions:

(a) A high concentration of CaM is a condition that enables CaM to operate at low Ca^{2+} concentrations.

(b) A high affinity of CaM for its target proteins can elevate the apparent affinity of CaM for Ca^{2+}.

(c) In addition to the above two items, it is possible that a ligand would increase the affinity of CaM for Ca^{2+} by changing its conformation directly or indirectly.

It has been shown that CaM regulation is rather tissue specific in spite of its common character. Since the content of CaM and its ligands in a cell differs very much from cell to cell, it is not unreasonable that modes of CaM action are different from tissue to tissue. In other words, the common nature of CaM itself enables the cell to express its specific features.

8. A new type of Ca^{2+} regulation

The search for the mechanism of CaM function has opened up the possibility of a new type of Ca^{2+} regulation, i.e., the increase in affinity of Ca^{2+}-binding protein for Ca^{2+} can be the mechanism of regulation. This increase can be induced by:

(i) Conversion of loose binding of Ca^{2+}-receptor protein to its ligand (target protein) into strong binding owing to the change in ligand, as suggested by item b in section 7. This change can be induced by a particular factor, but may also be brought about by an alteration in its environment, e.g., electrochemical gradient.

(ii) Increase in the affinity itself of Ca^{2+}-binding protein for Ca^{2+}. This can be induced by the above-mentioned mechanism (c), but also by the influence from the ligand. It is quite possible that the two mechanisms, (i) and (ii), are intermingled.

It has been stated that the intracellular Ca^{2+} concentration in a quiescent state is 10^{-7} M. A 10-fold increase in the affinity for Ca^{2+} may be sufficient for activating the system. Such a scale of increase is not far from being realistic in view of the data reported so far. If the factor producing the change in process i or ii is a chemical substance, then the next problem is how to remove this factor. A plausible mechanism for this is that the protein to which the factor binds is the enzyme itself to decompose the factor.

New Ca^{2+}-dependent reactions are now being found continuously, perhaps owing to progress in the techniques of detection of intracellular Ca^{2+} (these methods, particularly those using fluorescent dyes, must be examined more carefully, so as not to draw erroneous conclusions). In this connection, two factors must be noted. One is that Ca^{2+} concentrations lower than 10^{-7} M have often been noticed in relation to some physiological changes. The other is the discrepancy between the time course of Ca^{2+} concentration and that of the physiological activity, the former usually falling prior to the latter. In these cases, complicated modes of action of Ca^{2+} regulation must be taken into consideration, as emphasized in this article, before postulating a putative regulatory factor that is unrelated to Ca^{2+}.

9. Conclusion

Among the various kinds of factors related to Ca^{2+} regulation mechanisms, two are to be nominated as models. One is troponin, the most differentiated regulatory protein that can provide for the rapid and precise reactions underlying skeletal muscle contraction. The other is CaM, one of the most conservative proteins, being involved in various kinds of reactions in almost all tissues. In spite of such a common nature, CaM can exercise a number of functions specific to individual cells.

Ca^{2+} is one of the simplest chemicals in the living organism, but it is deeply involved in numerous cell activities of great diversity and its mode of action is remarkably complicated. Ca^{2+} regulation is, in a sense, a task for the cell itself in developing different kinds of complex devices for utilizing Ca^{2+} in a more efficient and elegant manner. Thus, the evolutionary aspect must be stressed more in future studies on Ca^{2+} regulation.

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