Journal of Pharmacological Sciences

Review

Calcium Ion as a Second Messenger With Special Reference to Excitation-Contraction Coupling

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Received March 3, 2006

Abstract. Calcium ion (Ca^{2+}) plays an important role in stimulus-response reactions of cells as a second messenger. This is done by keeping cytoplasmic Ca^{2+} concentration low at rest and by mobilizing Ca^{2+} in response to stimulus, which in turn activates the cellular reaction. The role of Ca^{2+} as a second messenger was first discovered in excitation-contraction coupling of skeletal muscle. The history of the discovery was reviewed. Characteristics of Ca^{2+} as a second messenger, diversity of target molecules, capability of rapid and massive mobilization and also of oscillatory mobilization, tendency toward localization, and on the other side, ability to cause generalized cell response were described. The possible bases for these characteristics was discussed. Ca^{2+} itself induces release of Ca^{2+} from the sarcoplasmic reticulum (Ca^{2+}-induced Ca^{2+} release [CICR]). The Ca^{2+} release channel, ryanodine receptor, incorporated into lipid bilayer shows CICR activity. Ca^{2+} release induced by inositol trisphosphate also has an apparent CICR nature. The significance of CICR or apparent CICR with its inherently regenerative nature in physiological contractions of skeletal, cardiac, and smooth muscles was discussed.

Keywords: calcium ion, second messenger, calcium-induced calcium release, excitation-contraction coupling, calcium oscillation

Introduction

Among elements constituting the human body, calcium is the fifth most abundant element after oxygen, carbon, hydrogen, and nitrogen. The total calcium content in the human body is about 1 kg, comprising some 1.5% of the body weight. The great majority of calcium in the body is present in bones and teeth, but the remaining 1% or so is distributed widely throughout the body and plays a very important physiological role.

Concentration of calcium ion (Ca^{2+}) in the cytoplasm is normally kept at a very low level, about 10^{-7} M, while that in the extracellular medium is of the order of 10^{-3} M. Inside the cell, there is a Ca^{2+} storage site, which is a membrane-bound organella that strongly accumulates Ca^{2+} to such an extent that its luminal Ca^{2+} concentration reaches millimolar level. Thus, there is a huge concentration gradient of Ca^{2+} across the surface membrane and membrane of the intracellular Ca^{2+} store. Some kinds of stimulation of the cell trigger a flow of Ca^{2+} from either the extracellular space or the intracellular Ca^{2+} store into the cytoplasm, usually by opening Ca^{2+} channels present in the boundary membrane to allow a passive Ca^{2+} movement down the electrochemical potential gradient. Ca^{2+} thus mobilized into the cytoplasm causes the response of the cell to the stimulus. When the stimulus ceases, Ca^{2+} is extruded back to the extracellular space or reaccumulated to the intracellular Ca^{2+} store to terminate the response. Active transport is necessary in this process because Ca^{2+} must move against the large electrochemical potential gradient. Thus, Ca^{2+} plays a role as a second messenger, which, in response to a stimulus delivered by a first messenger from outside, transmits the message of arrival of the stimulus to the intracellular system that carries out the response of the cell to the stimulus.

The term “second messenger” in the above sense was first used for cyclic adenosine monophosphate (cAMP) by Sutherland and Rall who discovered that it is pro-

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Published online in J-STAGE:
DOI: 10.1254/jphs.CPJ06004X

Invited article

duced in liver cells upon arrival of hormones, adrenaline or glucagon, and induces glucose production, the response of the cell, by activating phosphorylase (1). We now know that the molecular pathways from stimulus to the cellular response in general is not necessarily through a single second messenger, but sometimes more complex; two or more "messengers" may work in series in the pathway, for example. In this article the term "second messenger" is rather roughly used for a major low molecular substance that plays, on the pathway, an essential role in eliciting cell responses to a stimulus, even if it is not the "second" one, strictly speaking.

The stimulus-response reactions of the cell are vital for the living organism, and the cell uses a variety of second messengers for various reactions. Among those messengers, Ca^{2+} is one of the most important ones, and it was identified during the very early days, soon after cAMP, in excitation-contraction coupling (E-C coupling) of skeletal muscle. Following the discovery in skeletal muscle, diverse intracellular processes, including release of neurotransmitters and hormones, metabolic switching and other enzyme activations, gene expression, cell cycle progression, synaptic plasticity and so on, have been found to be regulated by Ca^{2+}.

In this article, the history of the establishment of the second messenger role of Ca^{2+} in skeletal muscle will be reviewed, and the characteristics of Ca^{2+} as a second messenger discussed. Then, Ca^{2+}-induced Ca^{2+} release (CICR), with which I have been concerned for a long time since its discovery, will be discussed in relation to E-C coupling in various types of muscles where Ca^{2+} plays the essential role as the second messenger.

Ca^{2+} as the key substance in E-C coupling

The importance of Ca^{2+} in contraction was first shown in 1883 by Ringer in cardiac muscle. He demonstrated that the presence of Ca^{2+} in the external medium is essential for contraction of cardiac muscle (2). However, this did not directly lead to the present concept of the role of Ca^{2+}. About half a century later, Heilbrunn demonstrated that a single skeletal muscle fiber with both ends cut contracts much more rapidly in the isotonic $CaCl_2$ solution than in physiological saline and claimed in 1940 that Ca^{2+} acts on the intracellular contractile elements (3). In 1943, Kamada and Kinoshita demonstrated the same fact in a more elegant and physiological way that injection of Ca^{2+} into intact single fibers through a micropipette induced reversible shortening of the part injected (4).

However, the concept that Ca^{2+} induces physiological contraction did not receive support at that time, especially among biochemists. This is because Ca^{2+} apparently exerted no effects at all on in vitro contraction, myosin-actin interaction in the presence of ATP. We know at present that experiments in those days were conducted unintentionally in the presence of a sufficient amount of Ca^{2+} that had come from glassware, slightly contaminated agents, and so on.

The present-day's concept that Ca^{2+} is the key substance in E-C coupling in skeletal muscle was established largely through the series of works by S. Ebashi. He was very much impressed by Szent-Gyorgyi's experiments described in his book "Chemistry of Muscular Contraction" published in 1949 that glycerinated muscle contracts upon addition of ATP, but at the same time he wondered why it does not relax even after the complete washout of ATP. He believed that there must be some factor in living muscle that induces relaxation of glycerinated muscle contracted by ATP and searched for the factor in homogenates of muscle tissues. In the meantime, such a relaxing factor was found and reported in 1951 by Marsh (5). Ebashi at that time in Kumagai's laboratory looked into further details of the factor and demonstrated in 1955 that it was nothing but a microsomal ATPase preparation (6), contrary to the general view of the time that it may be a soluble ATP-regenerating enzyme(s). As the mechanism of relaxation by the factor, a suggestive finding was reported already in 1954 that EDTA induces relaxation, which is reversed by the addition of Ca^{2+} (7). Ebashi extended it and showed in 1960 that Ca^{2+}-binding activity of chelating agents is strictly parallel to their relaxing activity under the physiological ionic condition (8). He then clearly demonstrated in 1961 for the first time by taking sufficient care to avoid contamination of Ca^{2+} that ATP-induced superprecipitation of natural actomyosin requires the presence of a minute amount of Ca^{2+}, of the order of 10^{-6} M (9). Furthermore, he showed that the relaxing factor is the fragmented sarcoplasmic reticulum (SR) and that it strongly accumulates Ca^{2+} in the presence of ATP and therefore, removes Ca^{2+} from the medium (10). Contraction is the reverse of relaxation, and Ebashi first suggested the present scheme of E-C coupling in 1961 that action potential evoked at the surface membrane somehow sends a signal to the SR to cause release of Ca^{2+} that had been accumulated during relaxing phase and the resting period.

Weber independently discovered the requirement of Ca^{2+} for ATPase activity of natural actomyosin (11). Hasselbach and Makinose also showed that the relaxing factor accumulates Ca^{2+} in the presence of ATP (12).

Ebashi then inquired into the mechanism of Ca^{2+} action on natural actomyosin: he discovered in 1963 that purified myosin and actin did not require Ca^{2+} for

superprecipitation and that a protein factor similar to tropomyosin, which is included in natural actomyosin preparation, is indeed necessary for the Ca^{2+} effect to be obtained (13). Later in 1965, the protein factor was found to be a complex of tropomyosin and a new protein, troponin (14). The Ca^{2+}-binding component of troponin, troponin C, is the Ca^{2+}-receptive protein in the contractile system.

The whole concept that Ca^{2+} is the second messenger in E-C coupling of skeletal muscle was outlined in some detail first in a review, "Ca ion and muscle contraction", which was published in 1968 (15).

Diversity of Ca^{2+}-regulated functions

Ebashi thought that the regulatory role of Ca^{2+} may be more general among cellular functions, and indeed it was shown in his laboratory as early as in 1967 that phosphorylase b kinase, the key enzyme of glycogenolysis, is activated by micromolar level of Ca^{2+} (16). The discovery of calmodulin, the Ca^{2+} receptive protein homologous to troponin C, by Cheung (17) and by Kakiuchi (18) around 1970 actually expanded the Ca^{2+} studies to tissues other than muscle, and following inumerable studies firmly established that Ca^{2+} is a universal second messenger in the eukaryotic cells.

How can a single substance, Ca^{2+}, regulate a variety of functions universally, while characteristics of the functions to be regulated (viz. speed, duration, intensity, and so on) are so diverse? Consider contractile responses for instance. Speed and duration of physiological contractions of skeletal muscle and those of smooth muscle could be orders of magnitude different. Also, in skeletal muscle force development in twitch responses of each fiber is approximately constant at a near maximal level, and regulation of force developed by the whole muscle is made by altering the number of fibers excited (and by altering firing frequency). In cardiac muscle, on the other hand, force developed by the cell is adjusted over a wide range to meet the physiological requirement, because all the cells contract in every beat so that regulation must be made at each cell level. These are only a few examples, and every kind of cell other than muscle also uses Ca^{2+} in their own way, so that characteristics of responses regulated by Ca^{2+} are really widely diverse.

To fulfill such diverse functions, nature created a wide variety of proteins for each step of Ca^{2+} handling and Ca^{2+}-induced reactions in each cell, and each cell is equipped with a set of proteins most appropriate for its own function. Thus, each cell uses a suitable Ca^{2+} source(s), either extracellular medium, intracellular Ca^{2+} store, or both, and has the most suitable channel protein(s) in the boundary membrane with its specific activating system and the most suitable Ca^{2+} active transport system(s). The same applies to Ca^{2+} receptive proteins and the following processes that lead to manifestation of function.

It was thought at first sight that if Ca^{2+} is a universal second messenger, Ca^{2+}-related drugs could not be found because of lack of specificity, which is essential for useful drugs. However, as described above, since each cell uses Ca^{2+} with its own specific proteins for each step of its Ca^{2+}-regulated functions, drugs targeting one of the specific proteins can, at least theoretically, be discovered. In fact we have such a useful group of drugs as calcium antagonists.

Characteristics of Ca^{2+} as a second messenger

Related to the diversity discussed in the previous section, direct molecular targets of Ca^{2+} as a second messenger, Ca^{2+}-receptive proteins, are far more diverse than those of other second messengers. The latter is usually single or only a few (for example, cAMP-activated protein kinase for most effects of cAMP), although many kinds of cell responses are regulated by the messenger-target protein complexes.

This difference between Ca^{2+} and other second messengers probably reflects the course of evolution. The composition of inorganic ions in the extracellular medium is similar to those of diluted seawater where life began; and life could have initiated only when the cell was able to establish its own intracellular ionic environment, including virtual absence of Ca^{2+}, entirely different from that of the extracellular medium. This uneven distribution of Ca^{2+} was utilized by cells for stimulus-response reactions as already described, and during the long course of evolution, a variety of ways of utilizing Ca^{2+} in this manner have been developed. This is quite different from other second messengers that were produced by living organisms much later in the history of evolution. Thus, the length of history of utilization of Ca^{2+} probably explains the extreme diversity of Ca^{2+}-regulated responses.

One of the significant characteristics of Ca^{2+} as a second messenger is its preexistence in great quantities on site, while other second messengers must be produced by enzymatic reactions after arrival of stimulus to the cell. Ca^{2+} can, therefore, be rapidly and massively mobilized simply by opening Ca^{2+} channels in the membrane separating the cytoplasm and Ca^{2+} sources, across which a huge electrochemical potential gradient of Ca^{2+} is present. This characteristic is quite suitable for skeletal muscle, which, following excitation, should quickly elicit nearly maximal response that requires a large amount of Ca^{2+} to activate the densely packed

contractile proteins. It is interesting that the role of Ca^{2+} as a second messenger was first discovered in skeletal muscle that fully utilizes this characteristic.

Another feature of Ca^{2+} as a second messenger is that Ca^{2+} mobilized by a stimulus could be confined to a small area. This is because there are many high-affinity Ca^{2+} binding sites in the cytoplasm, which retard and curtail diffusion of Ca^{2+} by trapping it. As a result, the effect of Ca^{2+} could be localized to a confined region, which is especially important in nerve cells, where localized responses such as synaptic plasticity are required.

On the other hand, Ca^{2+} is also used for generalized cellular response in which the entire part of the cell participates. Muscle contraction is usually a response of this type. If contraction is localized within a certain part of muscle fibers, force produced cannot be transmitted to the end of muscle. In order to elicit the synchronous response of whole cell with Ca^{2+} as the messenger, the diffusion of which tends to be restricted, cells are equipped with network of intracellular Ca^{2+} stores formed throughout the entire area of the cell. There are two types of mobilization of Ca^{2+} to induce generalized synchronous response with the network. In skeletal muscle, which must make a rapid response, Ca^{2+} mobilization from the store is elicited simultaneously everywhere throughout the cell with the aid of propagation of electrical response, action potential, as is well known. In smooth muscle, on the other hand, Ca^{2+} mobilization propagates from one part of the Ca^{2+} store to the neighboring region until it spreads throughout the cell as described in the next section. Although the propagation takes a certain time, the slow contractile reaction makes the synchronous response of the whole cell possible. Propagation of Ca^{2+} mobilization is also used in many cells other than muscle, where a generalized cell response or response to be transmitted from one site of the cell to the other is required.

Mobilization of Ca^{2+} could sometimes be oscillatory. This is probably unique among second messengers except possibly secondary oscillation of inositol trisphosphate (IP_3) simultaneous with that of Ca^{2+} (19), although the time course of second messengers other than Ca^{2+} in general has not been accurately followed. Physiological significance of oscillation of Ca^{2+} is not clear at present, but it is conceivable that since too much of Ca^{2+} is toxic to the cell, the cell might avoid a continuous presence of high concentrations of Ca^{2+} and use recurrent pulse-like mobilization of Ca^{2+}, whenever effective response can be obtained with it.

With these oscillations as well as propagation of Ca^{2+} mobilization on the one hand and tendency of localization on the other, spatiotemporal manifestation of Ca^{2+} could be infinitely various, which also constitutes the basis of diversity of effects of Ca^{2+} (20).

CICR from the SR

After Ebashi's achievement, the biggest problem to be solved in E-C coupling was the mechanism of Ca^{2+} release from the SR. During our investigation on Ca^{2+} releasing effect of caffeine, we found an interesting fact that Ca^{2+} itself induces release of Ca^{2+} from the SR (21). Ford and Podolsky also reported similar results independently (22). With this property, Ca^{2+} release could occur in an all-or-none like manner, since Ca^{2+} released would cause a further release of Ca^{2+}, a positive feedback loop being made. Therefore, at first we thought that this CICR might be related to the well-known sharp relationship between depolarization and tension in living skeletal muscle fibers. However, later we found that even if CICR was strongly suppressed by its inhibitors, procaine or adenine, K-contracture or twitch tension was not inhibited at all, indicating that physiological Ca^{2+} release in skeletal muscle is not mediated by CICR (cf. 23). CICR, however, operates in contractures in a pathological state known as malignant hyperthermia or in contractures induced by caffeine (23).

The Ca^{2+} release channel protein of the SR is known as ryanodine receptor (RyR), since it specifically binds an alkaloid, ryanodine, and by utilizing the specific binding the channel protein was isolated and purified. RyR consists of about 5000 amino acid residues with a membrane-spanning region towards its C-terminus end (24). It forms a tetramer that functions as a channel. Three types of RyRs are known, skeletal muscle type (RyR-1), cardiac type (RyR-2), and brain type (RyR-3). Purified RyR-1 as well as other types of RyRs incorporated into lipid bilayer membrane shows Ca^{2+}-channel activity with all the properties of CICR (25). RyR-1, therefore, is a CICR channel. On the other hand, the skeletal muscle of mice in which RyR-1 is knocked out, action potential or depolarization by K-rich solutions can no longer elicit contraction (26), indicating RyR-1 is the physiological Ca^{2+} release channel as well. Thus, one must conclude that RyR-1 channel can open in two different modes, physiological mode and CICR mode. Further studies are necessary to elucidate the exact molecular behavior of each mode of opening.

In contrast to skeletal muscle, it is generally believed that in mammalian cardiac muscle, Ca^{2+} that flows into cardiac cells during action potentials activates CICR channels of the SR to cause physiological Ca^{2+} release. Some problems had to be solved to accept the CICR theory of cardiac E-C coupling. First, CICR in cardiac skinned fiber under physiological condition appeared

not sensitive enough, the EC_{50} being about 2×10^{-6} M (23). Second, the rate of Ca^{2+} release was not uniquely determined by the intracellular Ca^{2+} concentration. Ca^{2+} release, begun upon depolarization, was immediately stopped by repolarization, whereas concentration of Ca^{2+} at that time had already reached the level sufficient to evoke Ca^{2+} release (27). Third, why could Ca^{2+} release be finely graded by Ca^{2+} influx despite of the positive feedback nature of CICR? The first two difficulties could be solved if the channel sees only the local Ca^{2+} concentration in the narrow space in the vicinity of the channel, which is different from the average Ca^{2+} concentration of the whole cell. The third problem could also be solved by a phenomenon called adaptation, a rapid negative control mechanism that terminates CICR while the trigger Ca^{2+} influx is still present (28).

It is interesting and rather ironical that CICR with its inherently regenerative nature is not used in skeletal muscle that releases Ca^{2+} in a quasi-all-or-none manner, but used in cardiac muscle that must release Ca^{2+} in a finely graded manner.

In smooth muscle, agonist-induced contractions are also caused by Ca^{2+} release from the intracellular Ca^{2+} store. In this case the Ca^{2+} channels responsible are those activated by IP_3, although RyRs are also present in smooth muscle cells. Iino found that the IP_3-receptor shows an apparent CICR nature; a sub-micromolar concentration of Ca^{2+} enhances channel opening (29), which plays an important role in agonist-induced contraction. It was shown that when concentration of agonist was gradually increased, Ca^{2+} release suddenly jumps from no response to maximal release. During the maximal Ca^{2+} release, the wave of Ca^{2+} release propagates throughout the cell utilizing the apparent CICR nature of Ca^{2+} release; Ca^{2+} released at a site enhances Ca^{2+} release at the neighboring site (30). Due to this propagation, approximately simultaneous activation of the contractile apparatus in whole cells is obtained to elicit effective tension production.

The self-regenerative nature of CICR, or apparent CICR, plays an essential role not only in addition to the propagation of Ca^{2+} release as described above but also in recurrent oscillatory Ca^{2+} release observed in muscles as well as many other kinds of cells. The spatiotemporal behavior of Ca^{2+} signal was reviewed in more detail by Iino (20).

Concluding remarks

It has been approaching half a century since the establishment of Ca^{2+} as a second messenger, and still the scope and depth of the research on Ca^{2+}-mediated cell responses seem to be growing. In this article due to the limitation of space, discussions with somewhat detailed facts and considerations were confined to muscle, and weight was given to the historical aspect. However, it is hoped that a certain essential nature of Ca^{2+}-mediated responses is expressed here.

Since Ca^{2+} is so essential in life, further cellular and molecular studies in this field will certainly broaden our scientific understandings of life. Further progress is awaited, especially since it will also form the basis of discovery of new Ca^{2+}-related drugs.

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